

# **IN-SITU BIOREDUCTION AND REMOVAL OF AMMONIUM PERCHLORATE**

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## Acronyms

DPRB	Dissimilatory (per)chlorate reducing bacteria
PCR	Polymerase chain reaction
<i>Pcr</i>	Perchlorate reductase
bls	Below land surface
CD	chlorite dismutase
IgG	Immunoglobulin
gm	gram
Kg	kilogram
ml	milliliter
mM	millimolar
$\mu$ M	micromolar
mmoles	millimoles
MPN	Most Probable Number
(per)chlorate	Referring to either chlorate and/or perchlorate
ppb	Parts per billion
TEAP	Terminal Electron Accepting Process



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## 1.0 Executive Summary

In recent years, perchlorate has become a household word for the American public as concerns about its presence in water supplies throughout the US have resulted in a public outcry. These concerns have been further fueled by articles in the popular press (125) recounting disputes between the US Environmental Protection Agency (EPA) and the Pentagon regarding the reporting and regulation of this contaminant (55, 91). Furthermore, the application of newly developed highly sensitive analytical techniques (117) has demonstrated that the true extent of perchlorate contamination was severely underestimated (24, 35, 78, 86) and the recent identification of its presence in major vegetable and dairy food products indicates that perchlorate might represent an even greater health threat than was previously considered (60)(URL: <http://www.ewg.org/reports/suspectsalads/>). Because of its unique chemical stability under environmental conditions and its high solubility remediation efforts for perchlorate in-situ have focused on microbial processes. Microbial respiration and reduction of perchlorate to chloride is a relatively recently recognized metabolism which offers great possibility for the successful development of robust remediation technologies. However, at the beginning of this project very little was known about this metabolism or the organisms involved. Our studies performed as part of our SERDP funded research and outlined in this report have investigated various aspects of the microbiology of perchlorate reduction. These studies have identified the ubiquity and diversity of organisms capable of this metabolism, identified the dominant perchlorate-reducing bacteria in the environment, investigated the biochemistry and genetics involved, and developed several new tools for application to the in-situ and ex-situ microbial treatment of perchlorate contamination. Many of the discoveries of these studies are responsible for the successful implementation of perchlorate bioremediation technologies and several of the tools developed are currently in commercial application. Overall, these studies have resulted in numerous articles in the popular press, several radio and television interviews, 31 publications in peer reviewed journals including two in *Nature* and one in *Nature Reviews Microbiology*, six patent applications, and more than 40 abstract/presentations at national meetings.

## 2.0 Objective

The objective of the studies described in this report were to gain a better understanding of the microbiology involved in microbial perchlorate reduction with an aim of enhancing the application of this novel metabolism to the attenuation of perchlorate contaminated environments.

## 3.0 Background

### 3.1 The Problem

Perchlorate ( $\text{ClO}_4^-$ ) is a soluble anion composed of a central chlorine atom surrounded by a tetrahedral array of four oxygen atoms. It is known to affect thyroid function in mammals(102) and its toxicity primarily results from its inhibition of thyroid hormone output. In addition, at

higher concentrations (6 mg per kg body weight per day) perchlorate can result in fatal bone marrow disease. Perchlorate binds to the sodium-iodide symporter and consequently competitively inhibits iodide uptake by the thyroid gland. Thyroid hormones are synthesized from iodide in the thyroid and are responsible for regulating mammalian metabolism. Longterm reduction in iodide uptake in an adult can ultimately result in hypothyroidism(25, 130). Furthermore, because the thyroid hormones are required for normal physical and mental development, exposure to thyroid inhibitors such as perchlorate may have a direct impact on fetal and infant neuropsychological development (56, 87). Previous studies have indicated that children of mothers suffering from maternal thyroid deficiency during pregnancy performed below average on 15 tests relating to intelligence, attention, language, reading ability, school performance and visual-motor performance (49).

Before 1997, perchlorate was an unregulated compound in the US. However, the discovery of perchlorate contamination in drinking water resources throughout the US especially those in the southwestern states of Nevada, Utah, and California prompted the establishment of a provisional action level of  $18 \mu\text{g.L}^{-1}$  in 1997 (92). In 1997, with the development of highly sensitive analytical techniques (129), perchlorate contamination of drinking and recreational waters was identified throughout the US and particularly in the southwestern states of Utah, Nevada, Colorado, and California. The California Environmental Protection Agency put in place a recommended maximum concentration limit (MCL) of  $18 \mu\text{g.L}^{-1}$  which if exceeded would require stoppage of water usage and remediation (92). This level was subsequently increased by the US Environmental Protection Agency to  $32 \mu\text{g.L}^{-1}$  (90), although most US states adhered to a value of  $18 \mu\text{g.L}^{-1}$ . In 1998 perchlorate was added to the US EPA Contaminant Candidate List for drinking water supplies (41) and in January 2002, as a result of the publication of a US EPA draft review on toxicological and risk assessment data associated with perchlorate contamination, a revised and lowered health protective standard of  $1 \mu\text{g.L}^{-1}$  was suggested (URL:[http://oaspub.epa.gov/eims/eimscomm.getfile?p\\_download\\_id=36247](http://oaspub.epa.gov/eims/eimscomm.getfile?p_download_id=36247)). In January of 2005 the US National Academy of Science reviewed the EPA draft report and suggested a revised maximum permissible dose of  $0.7 \mu\text{g kg}^{-1} \text{d}^{-1}$ . This suggestion correlates to a standard of  $\sim 23 \mu\text{g L}^{-1}$  for a normal adult person. However, the level would be lower for infants and children based on weight. This is especially poignant due to the recent study done on breast and dairy milk in the US. This study showed that perchlorate was detected in almost all milk samples analyzed. The highest level detected in breast milk was  $92 \mu\text{g L}^{-1}$ , a level 20 times higher than the NAS estimated maximum permissible dose for a baby. Reports of this magnitude are pressuring officials to set a final regulatory limit in the near future.

### 3.2 The Source

Perchlorate is principally a synthetic compound and its salts have a broad range of different industrial applications ranging from pyrotechnics to lubricating oils (78). Its presence in the environment predominantly results from legal historical discharge of unregulated manufacturing waste streams, leaching from disposal ponds, and from the periodic servicing of military inventories (115, 116, 127). The worst case was discovered in the Las Vegas, Nevada area where perchlorate has been manufactured for more than fifty years and groundwater contamination was discovered ranging from  $630,000 \mu\text{g.L}^{-1}$  to  $3,700,000 \mu\text{g.L}^{-1}$  (78). The only significant natural source of perchlorate known is associated with mineral deposits found in Chile where the perchlorate content averages as much as 0.03% of the total mineral mass (42).

Throughout the last century, the Chilean ore deposits were extensively mined as a mineral and nitrate source for fertilizer manufacture, and the perchlorate often persisted throughout processing into the final product at low concentrations (100, 119). Although the full extent of the historical use of Chilean ore-based fertilizers is unknown in the US, currently, their usage represents less than 0.2% of the fertilizer consumption in the USA (35). Furthermore, recent modifications to the refinement process have significantly reduced the perchlorate content of these products (119) and, as such, they are not thought to be a significant source of perchlorate in the environment (119).

By contrast, however, the presence of perchlorate has been indicated in a variety of other natural phosphorous-bearing minerals formed through evaporation processes (evaporites) collected from a diversity of arid locations(86). More recently, it was demonstrated that solid fertilizers not derived from the Chilean deposits and commonly used for the hydroponic growth of various fruit and vegetables can contain perchlorate at concentrations as high as 350 mg.kg<sup>-1</sup> (35). Such levels could represent a significant global health threat owing to the increasing use of hydroponic farming techniques for the production of a wide variety of plants for human consumption throughout the world (35). Studies performed on different plant species grown in soils containing perchlorate have indicated uptake (40, 108, 122) and in some cases transformation — reduction to chlorate (ClO<sub>3</sub><sup>-</sup>), chlorite (ClO<sub>2</sub><sup>-</sup>), and chloride (Cl<sup>-</sup>) — in the plant tissues (108, 122). In certain plant species such as tobacco and lettuce the perchlorate accumulates and persists during processing into the final shelf products, such as cigarettes, cigars and chewing tobacco, at concentrations as high as 60 mg.kg<sup>-1</sup> (40).

### 3.3 The Solution

As these sorts of studies continue and more sensitive analytical methods are developed (117), it is anticipated that other natural sources of perchlorate will be identified. For example, recent reports have indicated low-levels of perchlorate in drinking water wells in the southern part of Texas that exceeds an area of 30,000 square miles (24). This perchlorate is known not to be associated with industrial activities or the application of agricultural fertilizer, suggesting that it might also originate from an unidentified natural geological source (24). However, anthropogenic sources such as that found in Henderson, Nevada (55), which resulted in a major contaminant plume containing more than 9,000 metric tons of perchlorate (55) stretching down through the Las Vegas Wash, into Lake Mead and the Colorado River, will probably remain the principal culprit for the presence of perchlorate in water supplies. As of April 2003, perchlorate was manufactured and used in more than 150 industrial facilities throughout the US and more than 90 perchlorate releases have been reported in 25 states.

Ammonium perchlorate (NH<sub>4</sub>ClO<sub>4</sub>) represents approximately 90% of all perchlorate salts manufactured (78). It is predominantly used by the munitions industry and the US Defense Department as an energetics booster or oxidant in solid rocket fuels (78, 97, 114, 116). Although a powerful oxidant, under most environmental conditions perchlorate is highly stable and non-reactive owing to the high energy of activation associated with its reduction (115, 116). Because of the large molecular volume and single anionic charge, perchlorate also has a low affinity for cations and as a result, perchlorate salts, such as ammonium perchlorate, are generally highly soluble and completely dissociate into NH<sub>4</sub><sup>+</sup> and ClO<sub>4</sub><sup>-</sup> in aqueous solutions. Furthermore, perchlorate does not sorb to any significant extent to soils or sediments and, in the absence of

any biological interactions, its mobility and fate are largely influenced by the hydrology of the environment (118).

Because of its unique chemical stability and high solubility, remediation efforts for perchlorate contamination have focused primarily on microbial processes (116) and many novel bioremediative technologies are currently being developed (131). In 2001, a report published by Ground Water Remediation Technologies Analysis Center (97) outlined 65 different case studies of perchlorate-treatment technologies to target contaminated wastewater, surface water, groundwater and soils. The majority (45 case studies) were either in-situ or ex-situ biological treatment technologies based on the unique ability of some microorganisms to reductively respire perchlorate completely to innocuous chloride in the absence of oxygen (97). Other physical/chemical technologies such as adsorption by activated charcoal, reverse osmosis have proved difficult, or inapplicable, or have failed because of rapid saturation of active sites or high costs, especially those that are associated with the processing of surface or groundwater contamination where excessively large volumes containing low levels of perchlorate can require treatment. Although ion exchange technologies do show promise, they are non-selective and still require subsequent disposal of the removed perchlorate.

### 3.4 The Microorganisms

It has been known for more than fifty years that microorganisms can reduce oxyanions of chlorine such as chlorate ( $\text{ClO}_3^-$ ) and perchlorate ( $\text{ClO}_4^-$ ) [(per)chlorate] under anaerobic conditions (8). The high reduction potential of (per)chlorate ( $\text{ClO}_4^-/\text{Cl}^-$   $E^\circ = 1.287$  V;  $\text{ClO}_3^-/\text{Cl}^-$   $E^\circ = 1.03$  V) makes them ideal electron acceptors for microbial metabolism (32). Early studies indicated that unknown soil microorganisms rapidly reduced chlorate that was applied as a herbicide for thistle control (8) and the application of this reductive metabolism was later proposed for the measurement of sewage and wastewater biological oxygen demand (20, 21). Initially it was thought that chlorate reduction was mediated by nitrate-respiring microorganisms in the environment with chlorate uptake and reduction simply being a competitive reaction for the nitrate reductase system of these bacteria (39, 46, 48). This was supported by the fact that many nitrate-reducing microorganisms in pure culture were also capable of reducing (per)chlorate (39, 48, 95). Furthermore, early studies demonstrated that membrane-bound respiratory nitrate reductases and assimilatory nitrate reductases could alternatively reduce chlorate (106) and presumably perchlorate. For many years, selection for chlorate resistance has been used as a screening tool to obtain mutants that are unable to synthesize the molybdenum cofactor required for nitrate reduction (79). However, in all cases chlorite ( $\text{ClO}_2^-$ ) was produced as a toxic end product of (per)chlorate reduction by nitrate-reducing bacteria and no evidence was provided that these microorganisms could grow using this metabolism.

The goals of the current project were to investigate the many facets of the microbiology involved in microbial perchlorate reduction, to identify the phylogenetic and metabolic diversity of these organisms, to identify the environmental variables that control their activity, and to develop the protocols and tools required for an in-situ bioremediative strategy for the complete removal of perchlorate and any organic co-contaminants.

## 4.0 Materials and Methods

### 4.1. Ubiquity of perchlorate reducing bacteria

To investigate the diversity and ubiquity of microorganisms involved in the microbial reduction of (per)chlorate, we enumerated (per)chlorate-reducing bacteria (DPRB) from a broad diversity of environments including pristine and hydrocarbon contaminated soils, aquatic sediments, paper mill waste sludges, and farm animal waste lagoons. In all environments tested, acetate-oxidizing DPRB represented a significant population ranging from  $2.31 \times 10^3$  to  $2.4 \times 10^6$  cells per gram of sample. In addition, we isolated thirteen (per)chlorate-reducing bacteria from these environments and characterized them both phenotypically and phylogenetically.

#### 4.1.1 Sources of soils and sediments.

Soil samples were collected from the top 6 cm of an uncontaminated soil in Thompson Woods on the Carbondale campus of Southern Illinois University and also from a hydrocarbon-contaminated soil at Tulsa Tape Incorporated, Carbondale, IL. In addition, sediment samples were collected from campus lake and farm swine lagoons, Southern Illinois University, Carbondale, IL; Potomac River, Pohic Bay, VA; Mississippi River, Chester, IL; South Dakota gold mine drainage sediment, Hotsprings, SD; and swamp lands, Reston, FL. All samples were freshly collected and transported directly back to the lab where they were immediately assayed for (per)chlorate-reducing bacteria.

#### 4.1.2 Medium and culturing conditions.

Standard anaerobic culturing techniques were used throughout (10, 57, 77). The medium was boiled under  $N_2$ - $CO_2$  (80:20) to remove dissolved  $O_2$  and then dispensed into anaerobic pressure tubes or serum bottles under  $N_2$ - $CO_2$ , capped with thick butyl rubber stoppers, and sterilized by autoclaving. The basal medium was the bicarbonate-buffered freshwater medium that had previously been used for culturing strain CKB (18). Unless otherwise noted, sodium salts of acetate and chlorate (10 mM each) were used as the electron donor and acceptor, respectively, which were added from sterile anoxic stocks.

Alternative electron donors were added from sterile anoxic aqueous stocks. Pure aromatic hydrocarbons (benzene, hexadecane, and toluene) were added directly (1 ml to 10 ml of medium). Electron acceptors were also added from anoxic aqueous stocks. Soluble Fe(III) was supplied as Fe(III) chelated with nitrilotriacetic acid (Fe(III)-NTA) (10 mM) (94). Mn(IV) was supplied as synthetic  $MnO_2$  that was prepared as previously described (68) to give a final concentration of 10-30 mM. Sulfur was supplied as a polysulfide solution prepared as previously outlined (128). All other electron acceptors were prepared as anoxic aqueous stocks of the sodium salts to give final concentrations of 10 mM.

#### 4.1.3 Isolation of DPRB.

(Per)chlorate-reducing enrichments were established by transferring 1 g subsamples from each of the freshly collected soil and sediment samples into 9 ml of prepared anoxic medium under a gas stream of  $N_2$ - $CO_2$ . Acetate (10 mM) was the electron donor and chlorate (10 mM) was the electron acceptor. Incubations were done at 30 °C in the dark. Positive enrichments were identified by visual increase in optical density and by microscopic examination. Once a positive

enrichment was established the (per)chlorate-reducing culture was transferred (10% inoculum) into 9 ml of fresh anoxic medium. Isolated colonies were obtained from transfers of positive enrichments by the standard agar shake-tube technique with acetate as the sole electron donor and  $\text{ClO}_3^-$  (10 mM) as the sole electron acceptor.

#### 4.1.4 Most probable number counts.

Numbers of dissimilatory (per)chlorate-reducing bacteria were determined by three-tube most probable number counts (MPN) with 10 mM acetate as the electron donor. The medium contained (in grams per liter):  $\text{NH}_4\text{Cl}$  (0.25);  $\text{NaClO}_3$  (1.03);  $\text{CH}_3\text{COONa}$  (1.36);  $\text{NaH}_2\text{PO}_4$  (0.60);  $\text{KCl}$  (0.1);  $\text{NaHCO}_3$  (2.5). Vitamins and trace metals were added (10 ml/L) from stock solutions prepared as previously described (18). MPN series were incubated at room temperature in the dark for 60 days prior to analysis. Positives in the MPN series were identified visually by increase in optical density and also by microscopic examination.

#### 4.1.5 Chlorite dismutase purification and activity determination.

Washed cell suspensions of each of the DPRB isolates were analyzed for chlorite dismutase activity using a Clark  $\text{O}_2$  electrode as previously described (18, 30). In addition, the chlorite dismutase enzyme was purified to homogeneity from the soluble fraction of lysed cell preparations of the previously described (18) (per)chlorate-reducing strain CKB. Lysed cell preparations were prepared from chlorate grown cells of strain CKB and strain WD with acetate as the electron donor. Cells were harvested by centrifugation (10000 x g, 10 min, 4 °C). The resultant cell pellet was resuspended in 14 mM phosphate buffer (pH 7.2) with 0.5 mM phenylmethylsulfonyl fluoride. The cells were broken by three passes through a French pressure cell at 20,000 psi and treated with Dnase (10 mg/ml homogenate). Lysed cells were centrifuged at 26,000 g for 5 minutes to remove cell debris and the resulting supernatant was fractionated into soluble and membrane-bound proteins by ultracentrifugation (110,000 x g, 1 hour, 4 °C). The enzyme was purified from the cell-free extract using sequential chromatography. Initially the cell-free extract was passed over an anion-exchange column of Q Sepharose, followed by a hydroxyapatite column and a hydrophobic column of phenyl Superose. Active fractions were pooled and concentrated using ultrafiltration (30 kD MW cutoff). A final purification of the concentrated enzyme was done using a gel filtration column packed with Superdex 200 (Amersham Pharmacia Biotech, Piscataway, NJ). Homogeneity of purification was checked using SDS-PAGE gel electrophoresis. At each step of the purification protocol, fractions collected from the various columns were assayed for specific activity of chlorite dismutation by microassay.

#### 4.1.6 Cytochrome content.

As a preliminary investigation into the cytochrome content of the (per)chlorate-reducing isolates, dithionite-reduced versus air-oxidized difference spectra were obtained on washed cell suspensions of acetate-chlorate grown cells suspended in anoxic bicarbonate buffer (2.5 g.L<sup>-1</sup>) sparged with  $\text{N}_2\text{-CO}_2$  (80:20, vol:vol).

The ability of potential electron acceptors to oxidize the c-type cytochromes was determined as previously described (34). Briefly, cell suspensions (2 ml) were placed into two sealed glass cuvettes under  $\text{N}_2\text{-CO}_2$ . The suspensions were bubbled with  $\text{H}_2\text{-CO}_2$  (80:20) for 2 min to reduce the cytochromes and then bubbled with  $\text{N}_2\text{-CO}_2$  for 1 min. An aliquot (0.5 ml) of an anoxic 2.5

mM stock solution of the potential electron acceptor in bicarbonate buffer was added to one cuvette and 0.5 ml of the anoxic bicarbonate buffer was added to the second cuvette. Difference absorbance spectra for the two treatments were recorded with a scanning spectrophotometer.

#### 4.1.7 16S rRNA gene sequencing and analysis.

Cells from 2-ml cultures of DPRB were harvested by centrifugation, resuspended in 40 µl sterile water, and lysed by the addition of 5 µl chloroform with a 10 min incubation at 95 °C. Primers specific to bacterial 16S rDNA (8F: 5'- AGAGTTTGATCCTGGCTCAG-3'; 1525R: 5'- AAGGAGGTGATCCAGCC-3') were used in a polymerase chain reaction (PCR) that consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.2 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 75 ng of each primer, 0.5 µl Taq polymerase (Gibco/BRL), and 1 µl of lysed cells in a 50 µl reaction. Amplifications were performed at these parameters: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min with a final incubation of 10 min at 72 °C. The amplification products were gel-purified (GeneClean II, BIO101) and cycle sequenced (ThermoSequenase, Amersham) using internal primers. Some of the amplification products were cloned (TOPO TA cloning kit by Invitrogen) and then sequenced. Sequence entry and manipulation was performed with the MacVector 6.1 sequence analysis software program for the Macintosh (Oxford Molecular). Sequences of select 16S rRNAs were downloaded from the Ribosomal Database Project (69) and Genbank (14) into the computer program SeqApp (43). DPRB 16S rDNA sequences were manually added to the alignment using secondary structure information for proper alignment. Distance, parsimony, and maximum likelihood analysis of the aligned sequences was performed on a Power Macintosh G3 using PAUP\* 4.0d65 (109). Bootstrap analysis was conducted on 100 replications using a heuristic search strategy to assess the confidence level of various clades.

#### 4.1.8 Analytical techniques.

Acetate concentrations were analyzed by HPLC with UV detection (Shimadzu SPD-10A) using a HL-75H+ cation exchange column (Hamilton #79476). The eluent was 0.016N H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.4 ml per min. Perchlorate, chlorate and chloride concentrations were analyzed by HPLC with conductivity detection (Shimadzu CDD-6A) using a PRP-X100 anion exchange column (Hamilton #79434). The eluent was 4 mM p-hydroxybenzoic acid in 2.5% methanol with pH adjusted to 8.5, and a flow rate of 2.0 ml per min. Growth of cultures on soluble electron acceptors was measured by increase in optical density at 600 nm. Oxygen concentrations from chlorite dismutation were determined by an O<sub>2</sub> electrode (YSI, model 5300). Chlorite dismutase enzyme activity was determined by microassay using horseradish peroxidase (Sigma Chemical Corp., MO) coupled to dianisidine as an electron donor. In the presence of chlorite a brown color is produced which can be read spectrophotometrically at wavelength 450 nm (J.D. Coates, unpublished data).

## **4.2. Diversity of perchlorate reducing bacteria**

Here we present an in-depth analysis of the twenty new DPRB organisms which form two distinct monophyletic groups within the Rhodocyclus assemblage. Two new genera, *Dechloromonas* and *Dechlorosoma*, were proposed for these β-subclass lineages which represent the predominant (per)chlorate-reducing bacteria in the environment. The type species and strains



for these new genera are *Dechloromonas agitata* strain CKBT and *Dechlorosoma suillum* strain PST, respectively.

#### 4.2.1 Sources of soils and sediments.

The organisms were isolated from samples collected from a broad diversity of environments as previously described (31) including pristine and contaminated soils, sediments, and waste sludges. The isolates were obtained using standard shake-tube technique (18) with acetate as the electron donor and chlorate as the electron acceptor. All cultures were maintained in both active liquid stocks as well as anaerobic frozen stocks in 10% glycerol at -70 °C.

#### 4.2.2 Medium and culturing conditions.

Standard anaerobic culturing techniques were used throughout (57). The medium was boiled under N<sub>2</sub>-CO<sub>2</sub> (80:20) to remove dissolved O<sub>2</sub> and then dispensed into anaerobic pressure tubes or serum bottles under N<sub>2</sub>-CO<sub>2</sub>, capped with thick butyl rubber stoppers, and sterilized by autoclaving. The basal medium was the bicarbonate-buffered freshwater medium that had previously been used for culturing strain CKB (18). Sodium salts of acetate and chlorate (10 mM each) were used as the electron donor and acceptor, respectively, which were added from sterile anoxic stocks.

#### 4.2.3 16S rDNA sequencing and analysis.

Polymerase chain reaction (PCR) and sequencing of the 16S rRNA genes was performed as previously described (31). Sequence entry and manipulation was performed with the MacVector 6.5 sequence analysis software program for the Macintosh (Oxford Molecular Group). Sequences of select 16S rRNAs were downloaded from the Ribosomal Database Project (69) and Genbank (14) into the computer program SeqApp (43). 16S rDNA sequences of DPRB were manually added to the alignment using secondary structure information for proper alignment (alignment available on request). Complete 16S rDNA sequences were generated for 14 DPRB strains. For the remaining six DPRB strains, partial 16S rDNA sequences were determined. Only those regions sequenced in all of the organisms (815 nucl.) were used in the subsequent phylogenetic analyses (included *E. coli* positions 434-767, 807-1182 and 1266-1362). Distance, parsimony, and maximum likelihood analysis of the aligned sequences was performed on a Power Macintosh G3 using PAUP\* 4.0 (109). Bootstrap analysis was conducted on 100 replications using a heuristic search strategy to assess the confidence level of various clades. GenBank accession numbers for sequences are as follows: *Treponema pallidum* (M88726), *Magnetospirillum gryphiswaldense* (Y10109), Isolate WD (AF170352), *Azospirillum brasilense* (Z29617), Isolate TTI (AF170353), *Comamomas testosteroni* (M11224), *Ideonella dechloratans* (X72724), Isolate FL2 (AF288771), Isolate FL8 (AF288772), Isolate FL9 (AF288773), *Dechloromonas agitata* str. CKB (AF047462), Isolate CL (AF170354), Isolate NM (AF170355), Isolate CL24+ (AF288774), Isolate CL24 (AF288775), *Ferribacterium limneticum* (Y17060), Isolate MissR (AF170357), Isolate CCO (AF288776), Isolate SIUL (AF170356), *Rhodocyclus tenuis* (D16209), *Rhodocyclus purpureus* (M34132), *Azoarcus evansii* (X77679), *Azoarcus denitrificans* (L33689), *Thauera selenatis* (X68491), *Azoarcus indigens* (L15531), *Duganella zoogloeoides* (previously *Zoogloea ramigera*, X74913), *Dechlorosoma suillum* str. PS (AF170348), Isolate SDGM (AF170349), Isolate Iso1 (AF170350), Isolate Iso2 (AF170351), Gill symbiont of *Thyasira flexuosa* (L01575), Isolate NSS (AF170359), *Pseudomonas stutzeri*

(U26415), Isolate PK (AF170358), Isolate CFPBD (AF288777), *Wolinella succinogenes* ATCC 29543 (M26636), and *Helicobacter pylori* (M88157).

#### 4.2.4 Mol% G+C analysis.

Analyses of the mol% G+C of the chromosomal DNA was performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) using the high pressure liquid chromatography method and conditions as previously described (75, 110). Calibration of the method was performed with non-methylated lambda DNA (Sigma Chemicals, St. Louis, MO) (75).

### **4.3. Environmental factors that control microbial perchlorate reduction**

To elucidate the environmental parameters that control microbial perchlorate respiration we investigated the reduction of perchlorate by the dissimilatory perchlorate-reducer, *Dechlorosoma suillum* under a diverse set of environmental conditions

#### 4.3.1 Source of Organisms.

The facultative (per)chlorate reducing bacteria, *Dechlorosoma suillum* strain PS (1, 76) and *Dechloromonas agitata* strain CKB (1, 18) were taken from our laboratory culture collection of perchlorate reducing bacteria where they have been maintained as frozen stocks at -70 °C.

#### 4.3.2 Growth medium and culture conditions.

Unless otherwise stated, the medium used was a modification of the basal medium (BM) previously described for perchlorate-reducing bacteria (18). The BM was modified by replacing the carbonate buffer with a 10 mM phosphate buffer (pH 7.0). Acetate was amended as the electron donor from a concentrated stock solution (1.0 M) of the sodium salt to give the desired final concentration. Sodium salts of perchlorate and nitrate were also added from stock solutions (1 M respectively) as suitable electron acceptors when required. For anaerobic serum bottle cultures the prepared medium was made anoxic by purging with oxygen-free nitrogen and the bottles were crimp sealed with thick butyl rubber stoppers under nitrogen headspace before being autoclaved at 121 °C for 20 min.

#### 4.3.3 Batch fermentation.

Experiments were carried out in a 6L automatically controlled (pH, temperature, and dissolved oxygen) batch fermentor (New Brunswick Scientific Co. Inc., Edison, NJ; Model: Bioflow 2000) with an operating volume of 5L unless otherwise stated. The vessel was filled with fresh medium containing the appropriate electron donor and acceptor respectively, was sealed with the stainless steel lid equipped with probes for monitoring pH, dissolved oxygen, and temperature, and autoclaved at 121 °C for 35 minutes. The pH meter was calibrated before autoclaving. Immediately after autoclaving, the fermentor was connected to the control systems and all the parameters were set to their desired value. The dissolved oxygen content of each culture was maintained by balancing the mixed ratio of air to oxygen-free N<sub>2</sub> gas. The premixed gas was then filtered through a sterile 0.2 µm filter and bubbled through the media. Dissolved oxygen content was monitored by a previously calibrated dissolved oxygen sensor. Anoxic sterile solutions of 0.5 M H<sub>2</sub>SO<sub>4</sub> and 1.0 M NaOH were pumped through automatic acid and alkali

pumps, respectively, of the pH controller to maintain the pH. After reaching steady state relative to the set parameters, 500 ml of a twenty-hour growth culture of the pertinent perchlorate-reducing organism was used to inoculate the vessel. The speed of agitation in anaerobic run was fixed at 400 rpm, whereas in the aerobic run, the lower and upper limits of the speed of agitation was 400 rpm and 650 rpm respectively to control the dissolved oxygen concentration at the desired level. All cultures were incubated at 37°C. Samples were collected anaerobically when required in sterile containers at regular time intervals for analysis.

#### 4.3.4 Chlorite dismutase activity determination.

CD activity in collected culture samples was determined in triplicate by mixing 0.8 mL of the culture sample with 0.2 mL of a freshly prepared aqueous sodium chlorite stock solution (20 mM) in a 1.5 ml eppendorf tube. At appropriate time points (0 – 1.5 mins) 100  $\mu$ L subsamples were collected and transferred immediately into 1.5 ml eppendorf tubes in an 80 °C water bath to stop the reaction. The residual chlorite content in the heat killed subsamples was determined by colorimetric microassay based on Horse Radish Peroxidase and ortho-dianisidine dye as previously described (31, 33).

#### 4.3.5 Analyses.

The concentration of perchlorate in samples was determined by ion chromatography coupled to suppressed conductivity using a Dionex IonPac AS 11 4x250mm column (Dionex Corporation, Sunnyvale, CA) with a 100 mM NaOH mobile phase at a flow rate of 1 mL min<sup>-1</sup>. The eluting perchlorate was then detected by a conductivity detector (Shimadzu model: CDD - 6A), that was suppressed with a Dionex ASRS-Ultra operating in external water mode. The suppressor controller was set at 300 mA for the analysis. With an injection volume of 200  $\mu$ L, and the Anion Self-Regenerating Suppressor operating with an external water source, the detection limit for perchlorate was 6.0  $\mu$ g L<sup>-1</sup>. Chlorate, chloride, nitrate, and nitrite in the culture medium were determined using a Dionex DX500 ion chromatograph (Dionex Corporation, Sunnyvale, CA) equipped with a GP50 gradient pump, CD20 conductivity detector, ASRS-Ultra for suppressed conductivity, and PeakNet 6 controlling software. An IonPac AS9-SC 4x250 mm column was used for analysis with bicarbonate buffer containing 2 mM sodium carbonate and 0.75 mM sodium bicarbonate at a flow rate of 2 (mL min<sup>-1</sup>) as the eluent. The SRS current was set at 100 mA for all the analysis. Acetate concentrations were determined by HPLC (Shimadzu, Model: SPD-10A), equipped with UV-VIS detector, at a wavelength of 210 nm using a HL-75H+ cation-exchange column (Hamilton, Model no. 79476) and a mobile phase of 0.016 N H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.4 mL min<sup>-1</sup>. Culture growth was monitored both by optical density (OD at 600 nm) using a spectrophotometer (Spectronic Genesis 5; Spectronic Instruments, Inc.; Rochester, N.Y.) and by total cells count using phase-contrast microscope as previously described (31).

### **4.4. Stable isotope content of perchlorate and its fractionation during microbial metabolism**

As part of this work we characterized the stable isotopic signature for the chlorine content of (per)chlorate and chlorate in a range of flashpowder and weedkiller samples to explore the use of the technique for characterization of those compounds potentially for forensic purposes. In

addition, we also investigated the ability of perchlorate reducing bacteria to fractionate the stable isotope content of perchlorate through reductive dissimilation. The samples selected for analysis are listed in Table 4.1.

Weedkiller samples were separated into different size fractions as outlined in table 4.1. In order to check for intrinsic heterogeneity of each sample, duplicate analyses were always done on two separate powder aliquots. To ensure optimum condition of analysis, but still using the minimum of material, each aliquot was taken to provide around 35  $\mu\text{mol}$  of Cl. According to the compound and the size of the fraction analyzed the number of grains increase with decreasing fraction size: one to three grains for the 1.0 mm fraction, around 5 to 10 for 425  $\mu\text{m}$ , 15-20 for 355  $\mu\text{m}$ , 20-30 for 250, and more than 30 for 180 to 150  $\mu\text{m}$ . Powders at 63  $\mu\text{m}$  or below are so fine that the number of grains was not determined. The mass of powder (typically 2 to 5 mg) was weighed using a Sartorius balance. Blanks were performed to estimate the contamination level of these procedures. The chlorate in weedkiller samples was prepared by the Fe(II) reduction method (outlined below). The resultant chloride is converted to  $\text{CH}_3\text{Cl}$  for mass-spectrometric measurement.

Flashpowders were prepared by the standard analytical method (Ader et al., 2001) outlined below where perchlorate is stoichiometrically reduced (greater than 98% yield) to chloride using alkaline fusion-decomposition. To avoid any misinterpretation with potential intrinsic heterogeneity of some samples, chlorate and perchlorate solutions were prepared using a large aliquot of sample, at least six times bigger than the minimum quantity of powder required to perform one analysis. Duplicate analyses of such solutions should produce reproducible results. The methods for analysis of chlorate and perchlorate compounds measured values to a precision of 0.05‰. Mass spectrometer analysis used standard seawater Cl run as samples to calibrate the reference  $\text{CH}_3\text{Cl}$ .

#### 4.4.1 Perchlorate reduction for isotopic analyses.

Aliquots of around 5 mg of each sample were dissolved in approximately 10-15 ml of pure water. The perchlorate solution was made alkaline with potassium carbonate (around 300 mg of  $\text{K}_2\text{CO}_3$  for 40  $\mu\text{mol}$  of  $\text{ClO}_4$ ) and evaporated to dryness in a nickel crucible. The later was then placed in a muffle furnace, heated to 600°C (in 45 minutes) and cooled to ambient temperature. The resulting salt was dissolved in water and acidified with nitric acid. After Ader et al. (2001), this technique is quantitative and gives stable isotopic composition measurements with a reproducibility of  $\pm 0.10$  ‰. Some steps of the procedure were carefully refined to ensure good yields, to avoid any contamination and to reduce a potential isotopic shift during the evaporation step. Thus, the alkaline perchlorate solution was first evaporated on a hot plate at a temperature around 70-80°C. Before dryness was reached, it was then placed overnight in the oven at 80  $\pm 1$ °C, to complete the evaporation step. After the alkaline fusion and the dissolution steps, enough concentrated nitric acid (69 %) was added (up to 1 mL) to completely degas into  $\text{CO}_2$  the remaining  $\text{CO}_3^{2-}$  from  $\text{K}_2\text{CO}_3$ . Then, co-precipitation of  $\text{Ag}_2\text{CO}_3$  could not occur during  $\text{AgCl}$  precipitation and produce contamination later.

#### 4.4.2 Chlorate reduction for isotopic analysis.

Aliquots of around 4 mg of each sample were dissolved in 2.5 ml of pure water to produce a 0.02 molar chlorate solution. Then, 2.5 ml of a 0.2 molar ammonium iron (II) sulfate solution in 2

molar sulfuric acid and 1 ml of concentrated sulfuric acid were added. The mixture was heated at 80°C for 10 minutes, and allowed to cool to ambient temperature. After Ader et al. (2001), this technique is quantitative and give stable isotopic composition measurements with a reproducibility of  $\pm 0.18\%$ .

The alkaline fusion method used to reduce perchlorate (Ader et al., 2001) was tested to reduce chlorate as well.

#### 4.4.3 Culturing conditions for isotopic analysis.

The ability of *D. suillum* to fractionate chlorine isotopes while growing on perchlorate was determined by culturing the organism at 37 °C in modified version of our basal medium from which the major chloride salts were removed and replaced with equivalent sulfate salts. Culturing was performed with acetate (10 mM) as the sole electron donor and perchlorate (10 mM) as the sole electron acceptor in a 6L automatically controlled (pH, temperature, and dissolved oxygen) batch fermenter (New Brunswick Scientific Co. Inc, Edison, NJ; Model: Bioflow 2000). A value of pH 7.0 was maintained through the automatic dispensation of anoxic sterile solutions of either 0.5 M H<sub>2</sub>SO<sub>4</sub> or 1.0 M NaOH as appropriate. The inoculum culture of *D. suillum* was prepared in an identical chloride-free medium except that nitrate (10 mM) replaced perchlorate as the sole electron acceptor. Growth was monitored by microscopic observation and optical density measurements at 600 nm. Perchlorate and chloride concentrations were determined on subsamples collected at regular intervals throughout the growth cycle by ion chromatography analyses as previously described (Chaudhuri et al., 2002). Ion chromatographic analysis of the basal medium components prior to inoculation indicated the presence of a minor chloride contamination (0.2 mM) resulting from the vitamin and mineral stock solutions which was expected as some chloride salts were used in the preparation.

### **4.5. Perchlorate contaminated site characterization**

#### 4.5.1 Source of sediments, groundwater and organisms

Groundwater and sediment samples were collected from two separate sites at the Longhorn Army Ammunition Plant in Texas (LHAAP) by Dr. Paul Hatzinger of Envirogen Inc. and shipped directly to SIUC. The first location (Site 16) was a groundwater plume that was downgradient from a capped landfill. Sediments were taken from 22 – 26 feet below land surface (bls) using a Geoprobe apparatus fitted with disposable polyethylene core liners. Groundwater was collected from an existing monitoring well located near the core sample site. The second site at LHAAP (Site 25G) was near a former perchlorate mixing facility. Sediment samples were collected by Geoprobe from a depth of 12 to 16 feet bls. Groundwater was again collected from a nearby monitoring well. The core samples collected from both sites were cut into 2-ft sections and fitted with end-caps. The cores and groundwater were shipped overnight on ice to the laboratory. In addition, surface soil samples were collected from site 25. At the Naval Weapons Testing facility at San Nicolas Island, CA, subsurface soil samples were collected at 3 foot intervals down to 9 feet from three separate locations [heavy contamination (H), medium contamination (M), and pristine (P)]. The soil samples were collected using a Bosch hammer flighted hand auger. Samples were encased in plastic sleeves, capped off to prevent contamination, and sealed before being transported back to the laboratory. They were

immediately used to study the geochemical composition of each sample and to determine the presence and number of DPRB.

#### 4.5.2 Media and culturing techniques

Standard anaerobic culture techniques were used in the laboratory (4, 29, 37). Circum neutral pH basal medium was boiled under N<sub>2</sub>:CO<sub>2</sub> (80:20) to remove any dissolved O<sub>2</sub> and then dispensed into either anaerobic pressure tubes or serum bottles under a N<sub>2</sub>:CO<sub>2</sub> headspace. The circumneutral pH basal medium used was a bicarbonate-buffered freshwater medium that had been used in our laboratory previously (7). Separate media had to be made to account for each site's natural pH; thus, the standard bicarbonate-buffered medium could not be used for acidic or alkaline samples. The following modifications to the basal medium above were implemented to obtain the desired pH range. For acidic basal medium (pH range of 4.5 to 6.6), 2-[N-Morpholino]ethane sulfonic acid (4.67gm·l<sup>-1</sup>) was used as the buffer; Tris hydroxymethyl HCl (2.21gm·l<sup>-1</sup>) plus Tris-(hydroxymethyl) aminomethane (4.36gm·l<sup>-1</sup>) was used for the basic basal medium (pH range of 7.8 to 9.0). As neither of these two media have bicarbonate in them, only N<sub>2</sub> was used to sparge O<sub>2</sub> from the medium and likewise was used to flush the headspace exclusively. All acidic and alkali media were adjusted using 1N stocks of either NaOH or HCl to achieve the desired pH. All tubes and bottles were capped with thick butyl rubber stoppers and autoclaved for sterility (20 min at 121°C). Unless stated otherwise, sodium salts of both acetate and perchlorate (5 mM of each) were used as the electron donor and acceptor respectively, and were added into the basal medium directly for most probable numbers (MPNs) or were added from sterile anoxic stock solutions for other experiments.

#### 4.5.3 Most Probable Number series

The numbers of dissimilatory DPRB in soil, sediment and groundwater samples were determined by the three-tube MPN counting method described previously (18). All results were expressed as the number of DPRB per gram (wet weight) of soil or sediment sample or per ml of liquid sample. The medium used was the type described above and modified for the desired pH of the MPN series. MPN series preparations were incubated at room temperature in the dark for 30 days before taking readings. Positive enrichments were identified by an increase in optical density (determined visually) and by examination of the culture using a light microscope.

#### 4.5.4 Isolation of DPRB

Enrichment cultures for perchlorate-reducing bacteria were taken from positive MPN tubes described earlier. Positive results were determined as described earlier in the MPN series section. Positive enrichments were transferred (1 ml inoculum) into 9 ml of the appropriate medium a minimum of three times before isolation. Acetate (5 mM) was the electron donor and carbon source, while perchlorate (5 mM) was the electron acceptor. Preparations were kept at room temperature (~25°C) and all were stored in the dark. The standard agar shake-tube method described previously (7, 57) was used to obtain isolated colonies from positive enrichments, using acetate (5 mM) as the sole electron donor and carbon source and perchlorate (5 mM) as the sole electron acceptor.

#### 4.5.5 Terminal Electron Accepting Process setup and analysis

Terminal Electron Accepting Process (TEAP) determinations were performed on soil and sediment from each site, as previously described (14). Ten grams of soil from each site was

anaerobically placed into each of five 25 ml bottles, capped with thick butyl rubber stoppers, and crimped. The first two bottles were inoculated with an anaerobic, sterile 1 M sodium molybdate stock to a final concentration of 20 mM. The other three bottles were inoculated with a similar volume of anaerobic sterile deionized water. All bottles were inoculated with a known amount of  $^{14}\text{C}$ -acetate, vortexed for 30 seconds, and 1 ml of headspace was checked periodically for  $^{14}\text{C}$ -gas production in a GC-8A Gas Chromatograph (Shimadzu) and detected with a GC-RAM radioactivity detector (Insus Systems, Inc.). All bottles were kept in the dark at room temperature.

#### 4.5.6 Perchlorate remediation of contaminated soils

Soils from Longhorn, Texas were tested to see if the addition of acetate would stimulate the acetate-oxidizing, perchlorate-reducing microbial populations to metabolize the indigenous perchlorate. Five grams of soil from each site was anaerobically placed into six 25 ml bottles, capped with thick butyl rubber stoppers, and crimped. The first three bottles were inoculated with 1 mM acetate and the other three bottles were inoculated with anaerobic, sterile deionized water. All bottles had five ml of anaerobic, sterile deionized water plus 1 mM perchlorate added to them to facilitate sampling of perchlorate levels. Perchlorate concentrations were determined by ion chromatography with conductivity detection (Shimadzu model CDD-6A instrument) by using an anion-exchange column (IonPac AS16 4-mm). Samples were diluted to a final concentration of 1 in 500, as chloride levels tend to interfere with this particular column. The eluent was 50mM NaOH, and the flow rate was 1.00 ml per min.

#### 4.5.7 Alternative electron donors and acceptors

A variety of alternative electron donors and acceptors were added to media from sterile, anaerobic stock solutions. Sugars tested included sucrose, glucose, fructose, lactose, maltose, and ribose (10 mM). Alcohols used were ethanol, methanol, and glycerol (10 mM). Hydrocarbons tested were benzene (180  $\mu\text{M}$ ), toluene (180  $\mu\text{M}$ ), p-cresol (1 mM), phenol (1 mM), benzoate (1 mM), and phenanthrene (0.6 mM). Octane (1 mM) was used to determine if alkanes could be degraded. Fe(III)NTA (20), Fe(III)citrate (34), Fe(III)pyrophosphate (10), and a polysulfide stock (6) were prepared as previously described. Fatty acids tested included palmitic (1 mM), butyric, valeric, isovaleric, hexanoic, and heptanoic acid (5 mM). All other stock solutions were sodium salt solutions with the exception of propionate, which is a hemicalcium salt solution. The amount of ferric iron, polysulfide, and salt solutions added were in concentrations of 5 to 10 mM, with the exception of selenate (1 mM).

#### 4.5.8 Cell suspension preparation

Cells were grown in 500 ml of the appropriate medium with acetate and perchlorate as the only electron donor and acceptor, respectively. The cells were allowed to grow until reaching the end of their exponential growth phase, anaerobically transferred to centrifuge bottles, and centrifuged at 9820 g for 10 minutes. The cell pellets were washed twice in a pH buffer appropriate for each strain. They were anaerobically resuspended in 1 ml of the same buffer, placed into a 10 ml serum vial, and sealed with a butyl rubber stopper under a  $\text{N}_2$  headspace.

#### 4.5.9 Chlorite dismutase activity assay

Each strain was tested for chlorite dismutase (CD) activity by performing a colorimetric microassay using a mixture of horseradish peroxidase (Sigma) and o-dianisidine as a color

indicator (18). In the presence of chlorite, a yellow color is produced that can be measured colorimetrically at a 450 nm wavelength using a spectrophotometer. Total protein concentrations were detected colorimetrically on the spectrophotometer at a wavelength of 595 nm using the Bradford assay (18) and the rate of chlorite dismutation was divided by the total protein concentration to yield a chlorite dismutase specific activity for each strain.

#### 2.5.10 Analytical techniques

Perchlorate concentrations were determined by ion chromatography with conductivity detection (Shimadzu model CDD-6A instrument) by using an anion-exchange column (IonPac AS16 4-mm). Samples were diluted to a final concentration of 1:500, as chloride levels tend to interfere with this particular column. The eluent was 50mM NaOH, and the flow rate was 1.00 ml per min. Nitrate, sulfate, fluoride, and chloride concentrations were determined by Susan M. O'Connor using ion chromatography with conductivity detection (Dionex DX-500 with a CD 20 conductivity detector) by using an anion-exchange column (IonPac AS9-SC 4x250mm). The eluent was 2mM Na<sub>2</sub>CO<sub>3</sub>/ 0.75mM NaHCO<sub>3</sub> and the flow rate was 2.0 ml per min. Ferrous and ferric iron concentrations were detected using a colorimetric ferrozine assay (34). The pH of each site was analyzed on site using a pHep 3 pocket-sized microprocessor pH meter (Hanna instruments) and verified in the laboratory using an Accumet model 50 pH/ ion/ conductivity meter.

#### 4.5.11 DNA extractions from soil and liquid cultures

Bacterial DNA from soil and groundwater samples were obtained following the protocol used in the FastDNA SPIN Kit for Soil (BIO 101, Inc.). Genomic DNA suspensions from cell cultures were obtained by microcentrifugation of 1.5 ml cultures of DPRB, resuspended in 40 µl of SIGMA double-processed tissue culture water, and lysed by adding 5 µl of chloroform and incubating the preparations at 95° C for 10 minutes. The microcentrifuge tube was spun down to remove chloroform and cell fractions from the upper aqueous layer containing the genomic DNA.

#### 4.5.12 PCR conditions and primers used

Genomic DNA was used with primers that were specific for bacterial 16S ribosomal DNA (18) or that were specific for regions in the 16S rDNA common for most of the known DPRB (L. A. Achenbach, data unpublished). These primers were used in 50-µl PCR mixtures that contained 36 µl double-processed tissue culture water (Sigma), 1.5 mM MgCl<sub>2</sub>, Mg-free thermophilic DNA polymerase, 1x buffer (Promega), each deoxynucleoside triphosphate at a concentration of 0.2mM, 62.5 ng of each primer, 0.25 µl of Taq polymerase, and 1µl of genomic DNA suspension. Amplification was performed by using a Perkin Elmer GeneAmp PCR System 2400 under the following conditions: 94° C for 3 min, followed by 30 cycles consisting of 94° C for 1 min, 55° C for 1 min, 72° C for 1 min, and a final step consisting of 10 min at 72° C. Amplified PCR products were run in either a 0.7% or 2% Low EEO grade electrophoresis gel made with 1x TAE buffer, depending on the expected size of amplified genomic DNA.

#### 4.5.13 Sequence analysis of the 16S rRNA gene

The 16S rRNA gene from pure cultures of each unique strain of DPRB were sequenced and analyzed as previously described (1), with the following changes: additional GenBank accession numbers for sequences represented in Fig. 9 are as follows: *Dechloromonas sp.* strain LT-1,



AY124797; *Dechloromonas aromatica*, AY032610; *Dechlorosoma* sp. strain AH, AY171616; *Thauera aromatica*, X77118; and *Escherichia coli*, J01859.

## 4.6. Immunoprobe for perchlorate reducing bacteria

### 4.6.1 Media and culturing techniques.

All freshwater DPRB cultures including *Dechloromonas agitata* strain CKB, *Dechloromonas aromatica* strain RCB, *Dechlorosoma suillum* strain PS, *Pseudomonas* strain PK, and *Dechlorospirillum anomalous* strain WD were maintained on basal freshwater medium previously described (18). The marine DPRB *Dechloromarinus chlorophilus* strain NSS was maintained on APW medium as previously described (31). All DPRB were grown with acetate (10 mM) as the electron donor and either chlorate or perchlorate (10 mM) as the electron acceptor unless otherwise stated. In addition, active cultures of *Pseudomonas stutzeri*, and *Escherichia coli*, were grown aerobically on the same basal freshwater medium as the DPRB from which the (per)chlorate was omitted. *Rhodocyclus tenius* was grown on RCVB medium as previously described (111).

In the laboratory standard anaerobic culturing techniques were used (10, 57, 77). The anoxic basal medium was prepared by boiling under N<sub>2</sub>-CO<sub>2</sub> (80:20, v/v) to remove dissolved O<sub>2</sub> and dispensed under N<sub>2</sub>-CO<sub>2</sub> (80:20, v/v) into anaerobic pressure tubes or serum bottles that were then capped with thick butyl rubber stoppers and sterilized by autoclaving (15 min at 121 °C). Cell lysates were prepared from 1 L cultures of the various organisms as follows. The cells were harvested and resuspended in 5 mL of 10 mM phosphate buffer pH 7.3 and lysed by passing through a French press at 20 kpsi. The cell lysates were kept frozen at -80 °C until required. The protein concentration of both cell lysates and whole cells was determined using a Total Protein Determination kit from Sigma, St Louis MO. The cell lysates and whole cells were probed with the CD-specific IgG using the dot blot protocol outlined below. The lysates and whole cells were diluted such that each spot contained 5µg protein.

### 4.6.2 CD purification.

The chlorite dismutase was purified from the soluble fraction of a lysed cell preparation of *D. agitata* as previously described (31). Briefly a 30 ml sample of cell-free extract was loaded onto a 2.5 x 10 cm column packed with Q-sepharose fast flow (Amersham Pharmacia Biotech, Piscataway, NJ) media and the column was developed using a 0 to 300 mM KCl gradient in 50mM Tris-HCl pH 7.5. The CD active fractions were pooled and loaded onto a 2.5 x 20 cm column packed with hydroxyapatite (Bio-Rad Laboratories, CA) and developed with a potassium phosphate buffer gradient (10 to 250 mM, pH 7.2). The resulting CD active fractions were pooled and augmented with ammonium chloride to a final concentration of 2 M NH<sub>4</sub>Cl prior to loading onto a 1 x 1 cm phenyl sepharose high performance column. The phenyl sepharose column was developed with a descending gradient of ammonium chloride (2 to 0 M) in 50mM Tris-HCl, pH 7.5. The eluted chlorite dismutase was concentrated by ultrafiltration (MW cutoff of 30 kD) and passed through a 1.6 x 60 cm column packed with Superdex 200 media (Amersham Pharmacia Biotech, Piscataway, NJ). The pure chlorite dismutase was eluted with 150 mM NaCl in 50 mM potassium phosphate buffer, pH 7.2 and stored at -20 °C until used.

#### 4.6.3 CD-specific IgG.

Polyclonal antibodies to purified CD were commercially raised by Capralogics Inc., Hardwick, MA following a standard operating procedure for the production of rabbit polyclonal antibodies. The antigen was prepared by mixing CD with complete Freund's adjuvant and immunizing New Zealand type rabbits with 400 µg of the antigen by subcutaneous injection. Further boosts of 200 µg antigen were provided every three weeks. The initial test bleed four weeks after the first immunization indicated the presence of CD-active polyclonal antibodies. The rabbits were bled out after twelve weeks and the CD polyclonal antibodies were partially purified from the antiserum by ammonium sulfate precipitation (51). One volume of a saturated solution of  $\text{NH}_4\text{SO}_4$  was added to two volumes of antiserum, to precipitate the polyclonal antibodies. The resulting pellet was resuspended in 0.25 volume of phosphate buffered saline (PBS) and reprecipitated with one volume of saturated  $\text{NH}_4\text{SO}_4$ . The resulting IgG pellet was resuspended in 0.5 volume PBS and was dialyzed for 24 hours against PBS. The protein concentration of the dialysate was determined by the Bradford assay and the purified CD polyclonal IgG was stored at  $-80^\circ\text{C}$  in PBS at a final concentration of 15mg/mL.

#### 4.6.4 Dot-blot.

Dot-blot of the CD-specific IgG were prepared by spotting strips of Immobilon P transfer membranes (Millipore, Bedford MA) with diluted pure CD (1 µL of a 5 µg/µL), and air-dried. The membranes were then blocked for 1 hr in 0.1% TWEEN 20 PBS (TPBS) and incubated with the CD-specific IgG for 1 hr. The strips were washed 4 times in TPBS, (5 min per wash) and were further incubated with Goat antirabbit IgG (Sigma, St Louis MO) labeled with horse radish peroxidase (HRP) for 1hr in TPBS. The strips were washed as before and exposed to the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford IL), for 3 min, after which the luminescence was recorded using Kodak BioMax film.

#### 4.6.5 Western blots.

Pure CD and cell lysates from both *D. agitata* and *Escherichia coli* were run at 5mA over night on a 12% SDS-PAGE gel prepared as previously described (31) after which the gel was blotted onto Immobilon P transfer membrane using Towbin buffer (192mM Glycine:25mM Tris base:20% methanol pH 8.3) and a semi-dry transfer apparatus (Biorad, Hercules CA, Trans-Blot SD-Dry transfer cell) set at 20V for 20-30 mins. The resulting blot transfer was developed as outlined above in the dot-blot protocol while the post-transfer gel was stained with Coomassie Blue.

#### 4.6.6 Fluorescent micrographs.

Dry mounts of either *D. agitata* or *E. coli* were prepared for fluorescent microscopy by spreading a small volume (approx. 5 µL) of an active liquid culture onto an alcohol-cleaned glass slide. The slides were air dried at  $40^\circ\text{C}$  and rinsed briefly in PBS (0.05 M, pH 7.2). The primary antibody (rabbit polyclonal) was then applied at a dilution of 1:50 (diluted in PBS) and the slides were incubated for 30 min at room temperature after which the slide was again rinsed in PBS. The rinsed slides were then placed in a Coplin jar filled with PBS for an additional 10 min. After draining the slides of excess PBS, the fluorescein-labeled secondary IgG (goat anti-rabbit) was applied at a dilution of 1:100 and allowed to stand for 30 min at room temperature. The slide was rinsed with PBS as above and a coverslip was placed on the sample using a mountant composed of 9 parts glycerol and 1 part PBS (pH 8.5). The prepared slides were examined in a

Leica Orthoplan 2 epifluorescence microscope system and images were recorded using conventional photographic procedures or a Pixera 600CL Peltier-cooled CCD system.

#### **4.7. Identification, characterization, and classification of genes encoding perchlorate reductase**

##### 4.7.1 Growth conditions.

*D. agitata* and *D. aromatica* were grown both anaerobically and aerobically in basal media as previously described (11, 19). For anaerobic cultures, 10 mM acetate and 10 mM of perchlorate, chlorate, or nitrate were used as the electron donor and acceptor, respectively. For aerobic growth, (per)chlorate was omitted and oxygen was added to the same basal media. To check for induction under aerobic conditions, 1 mM sodium nitrate, chlorate, or perchlorate were added to aerobically grown cultures.

##### 4.7.2 Nucleic acid extraction and mutant construction.

Both gDNA and RNA were extracted as previously described using the PUREGENE DNA isolation kit (Gentra Systems Inc., Minneapolis, MN) and RNawiz reagent (Ambion, Austin, TX) (11). The  $\Delta$ *pcrA* mutant of *D. aromatica* was constructed by replacement of the *pcrA* gene with a tetracycline resistant cassette as previously described (101).

##### 4.7.3 Sequence analysis.

DNA sequences obtained from *D. agitata* lambda library screening (11) as well as the complete genome sequencing of *D. aromatica*, courtesy of the Joint Genome Institute, (<http://www.jgi.doe.gov>), were submitted to BLAST analysis (5). DNA sequence manipulation was performed using MacVector sequence analysis software for the Macintosh (version 7.0; Oxford Molecular) and the Se-Al Sequence Alignment Editor v. 1.0 (Rambaut, University of Oxford).

##### 4.7.4 Hybridization analyses.

Northern blots were performed using the NorthernMax-Gly glyoxal-based system (Ambion) as previously described (11). For all growth conditions, 5  $\mu$ g of total RNA was loaded onto a 1% (wt/vol) glyoxal agarose gel. Following RNA transfer, the blot was hybridized at 50°C in Easyhyb hybridization solution (Roche Applied Science, Indianapolis, IN) with a digoxigenin-labeled probe corresponding to 436 bp within the 5' half of the *D. agitata pcrA* gene. This probe was generated via PCR at 55°C annealing temperature with the following primers: PR-750F: 5'-CGCGAAGGTAGTCAGCATCT-3' and PR-1185R: 5'-TCCATCCTGCAACTTGACCT-3'. For DNA slot blots, genomic DNAs from known DPRB along with non-perchlorate reducing close relatives were blotted as previously described (11). The blot was hybridized at 45°C with the same perchlorate reductase probe used in the Northern blot analysis.

##### 4.7.5 Phylogenetic analysis.

Protein sequences from the  $\alpha$ -subunits of known DMSO enzymes were obtained from the GenBank database (15) and aligned with the perchlorate reductase using the CLUSTALW 1.82 program (112). The phylogenetic tree was constructed in the PAUP\* v 4.0 program (Swofford, Sinauer Associates) using distance as the criterion and neighbor-joining as the drawing method.

#### 4.7.6 GenBank accession numbers.

The GenBank accession numbers for the *D. agitata* perchlorate reductase are *pcrA* (AY180108), *pcrB* (submitted), *pcrD* (submitted). The accession numbers for the protein sequences in Fig. 2 are SerB (Q9S1G9), DPRB (P60069), EbdB (CAD58340), DdhB (AAN46633), and NarH (CAD22070). The GenBank accession numbers for the 16S rDNA sequences of the organisms in Fig. 7 are as follows: *Dechloromonas agitata* (AF047462), *Rhodocyclus tenuis* (D16209), *Dechloromonas aromatica* (AY032610), *Dechloromonas* strain JJ (AY032611), *Dechlorospirillum anomalous* strain WD (AF170352), *Magnetospirillum magnetotacticum* (Y10110), *Pseudomonas* strain PK (AF170358), *Pseudomonas stutzeri* (U26415), *Dechloromonas chlorophilus* strain NSS (AF170359), *Azospira suillum* (AF170348), *Dechloromonas* strain LT-1 (AY124797), *Ideonella dechloratans* (X72724).

### **4.8. The chlorite dismutase gene of *Dechloromonas agitata*: Sequencing, transcriptional analysis and its use as a metabolic probe**

#### 4.8.1 Growth conditions.

Both *Dechloromonas agitata* and *Ideonella dechloratans* (ATCC 51718) were grown at 30°C on basal media (18) following standard anaerobic techniques (57). Oxygen was removed by boiling and cooling under an N<sub>2</sub>-CO<sub>2</sub> (80-20, vol/vol) gas phase. Acetate (10 mM) and either chlorate or perchlorate (10 mM) were added respectively as electron donor and acceptor from sterile anoxic stock solutions. Aerobic growth was achieved utilizing the same basal media with oxygen as the electron acceptor. *E. coli* strains XL1-Blue MRF' and SOLR (Stratagene, LaJolla, CA), used for library screening, were maintained aerobically on LB media supplemented with 0.2% maltose and 10 mM MgSO<sub>4</sub> at 32°C. SOLR excision reactions were plated on LB supplemented with 50 µg/ml ampicillin to select for the pBluescript vector (Stratagene).

#### 4.8.2 Nucleic acid isolation.

Genomic DNA (gDNA) was extracted using the PUREGENE DNA Isolation Kit (Gentra Systems Inc., Minneapolis, MN). For RNA extractions, mid-log phase cultures were filtered and RNA was extracted using Lysing Matrix B tubes (BIO 101, Inc., Carlsbad, CA) and RNeasy reagent (Ambion, Austin, TX). To assess the level of RNA degradation, samples were electrophoresed on a 1.0% (wt/vol.) non-denaturing Tris-acetate-EDTA agarose gel.

#### 4.8.3 Southern Blot.

Five separate restriction enzyme digests were performed on *D. agitata* gDNA. Each digest was incubated at 37°C for 1 hr. and contained the following: 100 ng DNA, 1 µl buffer, 1 µl enzyme (*Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, or *Sac*I), and water to 10 µl. Enzymes and buffers were from Promega, Madison, WI. The digests were electrophoresed on a 0.7% agarose gel consisting of TAE. The gel was then denatured, neutralized, and blotted using standard techniques (99).

#### 4.8.4 Genomic library construction.

*Eco*RI-digested *D. agitata* total gDNA was electrophoresed on a 0.7% agarose gel consisting of TAE. Resulting fragments ranging between 3.0-8.0 kb were excised from the gel, purified (GeneClean II; BIO101) and ligated into Lambda ZAP II predigested *Eco*RI/CIAP-treated DNA

(Stratagene). Recombinant lambda vectors were packaged using the Gigapack III Gold packaging extract with *E.coli* strain XL1-Blue MRF' (Stratagene) as the host strain for library plating and amplification. Lambda ligation, packaging, and library amplification methods were all performed according to the manufacturer (Stratagene).

#### 4.8.5 Library screening.

Recombinant lambda DNA was transferred to nylon membranes in accordance with the manufacturer (Stratagene). PCR primers ICD-741F and ICD-1140R were utilized to construct a digoxigenin-labeled probe specific to 399 bases of the *Ideonella dechloratans* chlorite dismutase gene (Genbank Accession no. AJ296007; see Chlorite dismutase probe design). Membranes were hybridized at 46°C using 5 µl of probe per 10 ml of Easy Hyb solution (Roche Molecular Biochemicals, Indianapolis, IN) and detected using the Digoxigenin Luminescent Detection kit (Roche Molecular Biochemicals). Positive lambda clones were identified and used to isolate a phagemid containing the cloned *D. agitata* insert by in vivo excision (Stratagene).

#### 4.8.6 DNA sequencing and analysis.

Recombinant phagemid DNA was sequenced using the ThermoSequenase cycle sequencing kit (USB Corp, Cleveland, OH). A combination of vector-specific and insert-specific primers was used to completely sequence the *D. agitata cld* gene and flanking regions. Sequence entry and manipulation was performed using MacVector 7.0 sequence analysis software program for the Macintosh (Oxford Molecular) and the Se-Al Sequence Alignment Editor v1.0 (89). Protein sequence similarity was determined by BLAST 2.2 analysis (5).

#### 4.8.7 Primer extension.

Primer extension reactions with ( $\gamma$ -<sup>33</sup>P)ATP were performed using the Primer Extension System-AMV Reverse Transcriptase kit (Promega, Madison, WI). Primers specific to the 5' end of the CD mRNA were developed and used in primer extension reactions with 15 µg of total RNA from *D. agitata* cells grown under perchlorate-reducing conditions. Primer extension reactions were electrophoresed alongside sequencing reactions using the same primer on a standard 8M-urea 6.0% polyacrylamide gel for 3 hours at 39 watts.

#### 4.8.8 Chlorite dismutase probe design.

A probe corresponding to the 3' end of the *D. agitata cld* gene was labeled via PCR using the CD-394F and CD-756R primers (Table 4.2). Digoxigenin-11- dUTP (Roche Molecular Biochemicals) was incorporated by PCR reactions consisting of 35 µl tissue culture H<sub>2</sub>O, 5µl 10x PCR buffer, 3µl of 20mM MgCl<sub>2</sub>, 5µl of dNTP mix (13.2µl digoxigenin-11-dUTP at 1mM, 2.68µl dTTP at 10mM, 4ul each of dATP, dCTP, and dGTP at 10mM, 12.12 µl tissue culture H<sub>2</sub>O), 0.5µl of each forward and reverse primer at 125µg/ml, 1 µl of *D. agitata* lysed cells (1.5 ml of a turbid culture pelleted, resuspended in 40 µl of H<sub>2</sub>O and 5µl of chloroform, and heated at 100°C for 10 minutes) and 1 unit Taq DNA polymerase (67). All PCR reagents were from Promega. Reactions were cycled at the following parameters: 94°C for 3 min., followed by 30 cycles consisting of 94°C for 1 min., 55°C for 1 min., and 72°C for 2 min., ending with a 10 min. extension at 72°C.

#### 4.8.9 Northern blot analysis.

5 µg of total RNA from *D. agitata* cells grown under various conditions was electrophoresed on a 1.0% glyoxal-agarose gel and blotted according to the NorthernMax-Gly glyoxal-based system for northern blots manual (Ambion). Following transfer, the membrane was UV crosslinked (120 mJ/cm<sup>2</sup>) and hybridized according to manufacturer's instructions at 50°C with 5 µl of digoxigenin-labeled *D. agitata cld* probe per 10 ml of Easy Hyb solution (Roche Molecular Biochemicals). Detection was performed using the Digoxigenin Luminescent Detection kit (Roche Molecular Biochemicals).

#### 4.8.10 DNA slot blotting and screening.

250 ng of gDNA from previously isolated perchlorate- and chlorate-reducing strains and close relatives unable to reduce perchlorate were blotted onto Zeta-Probe blotting membrane using the Bio-Dot SF Microfiltration Apparatus (Bio-Rad, Hercules, CA). Following transfer, the blot was UV crosslinked (120 mJ/cm<sup>2</sup>) and hybridized at 46°C with the *D. agitata cld* probe following the same method as in the Northern blot analysis.

## 5.0 Results and accomplishments

### 5.1. Ubiquity and diversity of perchlorate reducing bacteria.

#### 5.1.1 Most probable number studies.

Most probable number counts with chlorate as the electron acceptor indicated that acetate-oxidizing, (per)chlorate-reducing bacteria are present in many diverse environments. The (per)chlorate-reducing microbial community represented a significant population in all environments tested, even those which have had no prior contact with oxyanions of chlorine (Table 5.1). The DPRB numbers ranged from  $2.31 \pm 1.33 \times 10^3$  to as high as  $2.40 \pm 1.74 \times 10^6$ . The highest MPN counts observed were in swine waste lagoons.

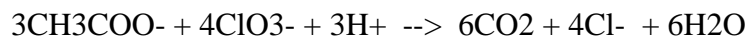
#### 5.1.2 DPRB isolates.

After two weeks incubation good growth was observed in the primary enrichments from all of the environments sampled. Enrichments were transferred into fresh basal medium (10% inoculum). Good growth was observed in the transfer after 24 hours as determined by increase in optical density and microscopic examination. Highly enriched (per)chlorate-reducing cultures were obtained by sequential transfer over the following week prior to serial dilution into agar tubes. Small colonies of consistent morphology were apparent in the higher dilution agar tubes from each enrichment after one week of incubation. Colonies were generally pink, wet, domed, entire, smooth, and small, being 1 to 4 mm in diameter. Several of these colonies were selected from each of the enrichment series and (per)chlorate-reducing isolates were obtained from all environments sampled.

#### 5.1.3 Phenotypic characteristics.

All of the DPRB isolates were complete-oxidizing, gram-negative, non-fermenting facultative anaerobes. Morphologically, most of the isolates were short motile rods 0.5 mm in diameter by 2 mm length. In contrast, some of the isolates, such as strain WD, which was a spirillum with cells 0.2 mm by 7 mm, represented alternative morphologies. Spores were not visible in wet-mounts of any of the isolates by phase contrast microscopy and no growth was observed in fresh acetate-chlorate medium after pasteurization at 80 °C for 3 minutes. All of the isolates could grow aerobically on L-broth and colonies on L-broth agar plates were generally white, smooth, and approximately 0.5 mm in diameter.

All of the DPRB isolates were strict respirers and could not grow on anoxic basal media amended with glucose (10 mM), yeast extract (10 g/l) and casamino acids (10 g/l). All of the DPRB isolates could couple the complete oxidation of acetate to the reduction of chlorate in defined basal medium (Fig. 5.1). The increase in cell numbers coincided with the oxidation of acetate and the production of chloride (Fig. 5.1). Chlorate was reduced to innocuous chloride for all isolates tested (strains PK, WD, NSS, and PS). The oxidation of 7.9 mM acetate resulted in the reduction of 9.3 mM of chlorate giving a stoichiometry of 1.18, which when assimilation into biomass is considered is in close agreement with the theoretical value according to:



Chlorite, the potential intermediate of chlorate reduction, was not detected in the culture broth. In addition to acetate, the DPRB isolates tested used short chain volatile fatty acids and simple dicarboxylic acids as alternative electron donors (Table 5.2). None of the DPRB isolates could use H<sub>2</sub> or hydrocarbons as alternative electron donors, although some could use inorganic electron donors such as Fe(II) (Table 5.2). The DPRB isolates were relatively limited in the range of electron acceptors used. In addition to perchlorate, chlorate, and O<sub>2</sub>, some of the isolates, but not all, could use nitrate for anaerobic growth (Table 5.3). A broad range of alternative electron acceptors were not used by the DPRB isolates (Table 5.3).

#### 5.1.4 Cytochrome content and oxidation by potential electron acceptors.

Air oxidized minus dithionite reduced spectra of washed whole cell suspensions of all of the DPRB isolates grown with chlorate as an electron acceptor gave absorbance maxima close to 425, 525, and 552 nm which is indicative of the presence of c-type cytochrome(s) (Fig. 5.2). Hydrogen-reduced c-type cytochrome(s) in anoxic washed cell suspensions of one of our previously described isolates (strain CKB) (18) were reoxidized by compounds known to act as electron acceptors for this organism such as chlorate or perchlorate. Hydrogen-reduced cytochrome(s) were not reoxidized by compounds such as sulfate, Fe(III), or fumarate which are not used by this organism as electron acceptors for anaerobic growth (Fig. 5.2).

#### 5.1.5 Phylogeny of the DPRB.

Analyses of the 16S rDNA sequences indicated that all isolates were members of the Proteobacteria phylum of the Bacteria (Fig. 5.3). DPRB isolates belonged to three subgroups (alpha, beta, and gamma) of the Proteobacteria, demonstrating that this metabolism is widespread throughout the phylum (Fig. 3). Some of these isolates, such as strain PK, were closely related to previously described genera not recognized for the potential to grow by dissimilatory (per)chlorate reduction while others, such as strain NSS, have no close relatives and represent novel genera in the Proteobacteria (Fig. 3). The majority of the isolates obtained were closely related to each other and to the phototrophic *Rhodocyclus* species in the beta subclass of the Proteobacteria.

#### 5.1.6 Chlorite dismutase.

Washed whole cell suspensions of all of the DPRB isolates could dismutate chlorite to chloride and molecular oxygen. At room temperature, O<sub>2</sub> evolution was rapid, linear and proportional to the chlorite concentration added (data not shown). No O<sub>2</sub> production was observed in the absence of cells or if the DPRB being tested was heat killed. At higher chlorite concentrations (10 mM), O<sub>2</sub> evolution was so extensive that a copious quantity of froth was observed in the cell suspension.

A single enzyme with chlorite dismutase activity was purified to homogeneity from the previously characterized strain (strain CKB) (18) (Fig. 5.4). The specific activity of this enzyme was 3,823 mmol chlorite per mg protein per min. Comparison against molecular weight standards in the electrophoresis gels indicated a molecular weight of 32 kDa for the denatured protein and 120 kDa for the native protein suggesting that the enzyme is a homotetramer with a molecular weight in the order of 120 kDa.



#### 5.1.7 Relevance.

The results of this study demonstrate the hitherto unrecognized ubiquity of microbial (per)chlorate reduction and the broad phylogenetic diversity of the organisms capable of this metabolism. Contamination of drinking water, ground water, and surface water by oxyanions of chlorine, especially chlorate and perchlorate has only recently been recognized as a potentially serious health risk (92, 116, 120). Although microbial reduction of (per)chlorate has been recognized for the last 50 years and was identified as a potentially important metabolism for the treatment of perchlorate and chlorate contamination in the environment (116, 121, 126), there is still very little known about the microorganisms involved in (per)chlorate reduction. Several organisms have been shown to be capable of the reduction of chlorate to chlorite, including *Proteus mirabilis* (39), *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* (95), however, no growth is associated with this metabolism and the chlorite endproduct is generally toxic to these organisms. The most probable number counts done in this study indicate that microbial (per)chlorate reduction is prevalent in significant numbers in a broad diversity of environments, many of which have had no prior exposure to chlorine oxyanions. This result supports and further expands the observations of a previous investigation (124) in which it was shown that chlorate reduction was prevalent in several diverse environments. This is, however, unexpected as there are no known natural sources of these compounds (54) and they have only been introduced into the environment in the last hundred years due to human activities (116). Although early studies suggested that microbial (per)chlorate reduction may simply be a competitive reaction for the nitrate reductase system of denitrifying bacteria in the environment (46, 47, 107), this does not explain the presence of chlorate reductase enzymes such as the chlorate reductase C purified from *Proteus mirabilis* which can only use chlorate as a substrate (85).

Prior to this study, only six dissimilatory (per)chlorate-reducing organisms had been identified and only four of these were studied in detail. Thus, the true diversity of microbial (per)chlorate reduction was still unknown. This study significantly increases the number of (per)chlorate-reducing isolates described. All of the isolates obtained are members of the Proteobacteria and represent three of the five subclasses of this phylum. Although this study did not produce any DPRB isolates which are members of the epsilon or delta subclasses of the Proteobacteria, a previously described DPRB, strain HAP-1 (127) was identified as a new strain of *Wolinella succinogenes* which is a member of the epsilon subclass. Thus, the metabolic capability of (per)chlorate reduction is widespread throughout the Proteobacteria. The broad phylogenetic diversity of organisms observed in this study which are capable of this metabolism has some interesting evolutionary implications due to the relatively short time in which (per)chlorate reduction could have evolved.

Several of the isolates were representatives of previously defined genera not recognized for the capability of (per)chlorate reduction. When some of the known close relatives to the DPRB isolates such as *Pseudomonas stutzeri*, the closest known relative to strain PK (99.4 % similarity by 16S rDNA sequence analysis), were tested for (per)chlorate reduction, no growth was observed and none of these organisms could dismutate chlorite. This result is similar to previous observations made with the DPRB *W. succinogenes* strain HAP-1 which is 99.3% similar by 16S rDNA sequence analysis to the type strain of *W. succinogenes* ATCC 29543 which cannot grow by (per)chlorate reduction (127).

#### **5.1.7.1 Comparison with other DPRB.**

Many of the DPRB isolates obtained in this study were not closely related to any known organisms and represented new genera within the Proteobacteria. All of the DPRB isolates contained c-type cytochromes, which in the case of the previously described DPRB (18), strain CKB, were reoxidized in the presence of physiological electron acceptors used by this organism. Other compounds such as sulfate, fumarate, or Fe(III) which were not reduced by this organism in anaerobic culture, also did not reoxidize the reduced cytochrome(s). Although not conclusive, this data suggests that the c-type cytochromes may be involved in the transport of electrons to perchlorate or chlorate.

Of the other known dissimilatory (per)chlorate-reducers, only four, strain GR-1 (93), *Ideonella dechloratans* (70), *Wolinella succinogenes* strain HAP-1 (127), and strain CKB (18) have been well characterized. Strains CKB (18), GR-1 (93), and *I. dechloratans* (70) are members of the beta subclass of the Proteobacteria. *I. dechloratans* is phylogenetically distinct from any of the DPRB isolates obtained during this study. Similar comparisons with strain GR-1 could not be made as the 16S rDNA sequence for this isolate is not available. *W. succinogenes* strain HAP-1 is a member of the epsilon subclass of the Proteobacteria and as such is very distantly related to any of the DPRB isolates obtained (127). All of the DPRB isolates are similar to strain GR-1 (93) and *I. dechloratans* (70) in their ability to couple growth to the oxidation of acetate with chlorate serving as the sole electron acceptor. As previously observed with other dissimilatory (per)chlorate-reducers (70, 93, 127), chlorate is completely reduced to chloride.

Similarly to strain CKB (18) and in contrast to previously described (per)chlorate-reducers (70, 93, 105, 127), the new DPRB isolates were relatively limited in the range of electron donors or acceptors used. None of the isolates utilized carbohydrates which are used by *I. dechloratans* (70). In addition, none of the new DPRB isolates could oxidize H<sub>2</sub>, an important endproduct of fermentation which does serve as an electron donor for (per)chlorate reduction by *W. succinogenes* strain HAP-1 (127). The only electron acceptors of those tested utilized by the DPRB isolates were O<sub>2</sub>, perchlorate, chlorate, and in some cases nitrate. The ability of these isolates to grow aerobically is similar to the previously described (per)chlorate-reducing bacteria and suggests that all (per)chlorate-reducing bacteria are facultative anaerobes. This is further supported by the fact that all of the (per)chlorate isolates obtained dismutate chlorite into chloride and O<sub>2</sub> which would be toxic to strict anaerobes. Although it was originally suggested that *W. succinogenes* strain HAP-1 was a strict anaerobe (127), a recent study indicates that it is in fact a microaerophile (126). The fact that not all of the DPRB isolates can use nitrate further supports the hypothesis that chlorate reduction and nitrate reduction are two unrelated pathways and is in contrast to the suggestions of earlier studies (46, 48, 107).

All of the DPRB isolates contain chlorite dismutase activity. The transformation of chlorite by these isolates, like that of strain GR-1 (93), is not dependant on the presence of acetate. Similarly to strain GR-1, a single enzyme was responsible for the dismutation activity in strain CKB. The purified enzyme has a high specific activity which is in the same order of magnitude as that observed with the enzyme isolated from strain GR-1 (123).

### 5.1.7.2 Environmental significance.

The role of (per)chlorate-reducing bacteria in environments that have no previous exposure to chlorine oxyanions has yet to be determined. Although a few dissimilatory (per)chlorate-reducers have now been described (18, 70, 93, 105, 127), all of these isolates were obtained from contaminated sediments or wastewater treatment sludges. This study is the first demonstration that organisms with (per)chlorate-reducing capability can be readily isolated from pristine environments.

Although (per)chlorate reduction has been recognized for more than fifty years, the presence of oxyanions of chlorine in the environment is the result of human activities over the last hundred years. As such, the evolution of such a phylogenetically diverse group of organisms with the ability to couple growth to the reduction of (per)chlorate is unexpected. This metabolic capability appears to be centered around the unique ability of these organisms to dismutate chlorite into chloride and oxygen. Although chlorite dismutation has not yet been demonstrated for all the known dissimilatory (per)chlorate reducing bacteria, it was shown previously for strain GR-1 (93), strain CKB (18, 29, 30) and in this study for all thirteen of the DPRB isolates thus suggesting that it is common to all DPRB. The fact that the purified chlorite dismutase enzymes from strains GR-1 and CKB were similar in general structure, molecular weight, and specific activity suggests that a single gene encoding for this enzyme may be conserved amongst these (per)chlorate-reducing organisms and further suggests that (per)chlorate reduction may be the result of horizontal gene transfer events.

## 5.2. *Dechloromonas* and *Dechlorosoma* species

### 5.2.1 Phylogenetic analysis.

Although DPRB representatives can be found throughout the Proteobacteria (31, 32, 127), the current work demonstrates that the majority (15 strains) of the DPRB isolated to date for which 16S rDNA sequence data is available (22 strains) are closely related to each other and are classified within the Rhodocyclus assemblage in the subclass of the Proteobacteria (Fig. 5.5). Analyses of the 16S rDNA sequences indicated that these (per)chlorate-reducing isolates form two distinct monophyletic groups within the subclass (Fig. 5.5). One of these groups, represented by our previously published DPRB strain CKB (18), contains 11 strains and is relatively diverse with 16S rDNA distances among this group ranging from 0 – 3.9%. The other group is represented by strain PST (31, 32) and contains four isolates that are more phylogenetically compact than those members in the CKB group with distances only ranging from 0 – 0.12%. Representative DPRB from the CKB group are most closely related to the Fe(III)-reducer *Ferribacterium limneticum* (37) while PS group members are most closely related to the phototroph *Rhodocyclus tenuis*.

Based on the unique phenotypic (18, 31, 32) and genotypic characteristics of the organisms in this assemblage of DPRB, we propose that the two groups of DPRB within the subclass of the Proteobacteria represent two new genera within the Rhodocyclus assemblage. These are denoted *Dechloromonas* and *Dechlorosoma* for the CKB and PS group members, respectively. *Dechloromonas agitata* strain CKB (18) is the type strain for the *Dechloromonas* genus and *Dechlorosoma suillum* strain PST (32) is the type strain for the *Dechlorosoma* genus. Unlike other members of the *Rhodocyclus* assemblage, these organisms are capable of dissimilatory

(per)chlorate reduction and chlorite dismutation (18, 31, 32). In addition, 16S rDNA sequences between the species of the two groups within this assemblage of DPRB are similar by no more than 94.1%. For comparison, *R. tenuis* shares 94.7% 16S sequence similarity to the most similar *Dechloromonas* species (strain CKBT) and 94.0% similarity to the most similar *Dechlorosoma* species (strain Iso2). The mol% G+C for these two groups of DPRB is also distinct with *Dechloromonas agitata* str. CKBT at 63.5% + 0.3 mol% G+C and *Dechlorosoma suillum* str. PST at 65.8 + 0.2 mol% (mean + standard deviation, n=3). *R. tenuis* has a mol% G+C of 64.8% (113).

All of the (per)chlorate-reducing *Dechloromonas* and *Dechlorosoma* species are heterotrophic facultative anaerobic respirers (18, 31, 32). Other common features include the ability to couple growth to the complete oxidation of acetate with chlorate or perchlorate as the sole electron acceptor and the ability to dismutate chlorite into chloride and O<sub>2</sub> (31). Some of the isolates can alternatively use nitrate as an electron acceptor (31) and nitrate is completely reduced to N<sub>2</sub>. Chlorite dismutation is a central step in the enzymatic reductive pathway of dissimilatory (per)chlorate reduction that is common to all tested dissimilatory (per)chlorate-reducing bacteria (31). (Per)chlorate is completely reduced to innocuous chloride.

Ideonella dechloratans, the only other previously described (per)chlorate-reducer which has been shown to be a member of the  $\beta$  subclass of the Proteobacteria, was not closely related to either group and showed a maximum similarity of 90.1% to *D. agitata* strain CKBT. Although previous studies have stated that the DPRB strain GR-1 is also a member of the  $\beta$  subclass of the Proteobacteria (93), this has never been demonstrated and the 16S rRNA sequence data is not available for comparative analysis.

We had previously shown that *Rhodocyclus tenuis* is incapable of reducing (per)chlorate (18) and that washed whole cell suspensions of *R. tenuis* were incapable of dismutating chlorite into chloride and O<sub>2</sub>. None of the other close relatives to *Dechloromonas* sp. and *Dechlorosoma* sp. that were tested could grow and reduce (per)chlorate or dismutate chlorite in washed whole cell suspensions (data not shown and (31, 32).

Residing within the *Dechloromonas* group is a previously characterized Fe(III)-reducer, *Ferribacterium limneticum* (37), that shares 97.3% 16S sequence similarity to its closest relative, the DPRB strain MissR. Interestingly, *F. limneticum* is a strict anaerobic respirer unable to reduce (per)chlorate (data not shown). None of the DPRB isolated in this study were able to reduce ferric iron (31). In addition, similarly to *R. tenuis* and in contrast to all tested (per)chlorate-reducing bacteria, washed whole-cell suspensions of *F. limneticum* did not dismutate chlorite into chloride and O<sub>2</sub>. This is in contrast to all known DPRB, including the isolates described in this study, which are facultative anaerobes capable of coupling growth to the dissimilation of either perchlorate or chlorate (31). The distinct physiological differences between *F. limneticum* and the closely related DPRB imply that *F. limneticum* is in fact a member of a separate genus.

Although the DPRB *Wolinella succinogenes* strain HAP-1 was originally thought to be a strict anaerobe, a recent study indicated that it is, in fact, a microaerophile (126). This is similar to one of our isolates, “*Dechlorospirillum anomalous*” strain WD, a member of the  $\alpha$  subclass of the

Proteobacteria closely related to the magnetotactic *Magnetospirillum* species (32), which also grows preferentially under microaerophilic conditions.

### 5.2.2 Significance.

To date, very little is known about the microorganisms capable of dissimilatory (per)chlorate reduction and until recently only three organisms had been described both phenotypically and genotypically which are capable of this metabolism (18, 70, 127). Here and in previous work we describe several new (per)chlorate-reducing organisms that were isolated from a broad diversity of environments (31, 32). Sequence analyses of the 16S rDNAs from the isolates indicated that all were members of three of the five subclasses ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) of the Proteobacteria (31). A previously described (per)chlorate-reducing bacterium, *Wolinella succinogenes* strain HAP-1 (127), was shown to be a member of the  $\epsilon$  subclass. These results demonstrate that the phylogenetic diversity of dissimilatory (per)chlorate-reducing bacteria is far greater than was previously considered. Most Proteobacterial subclasses contain only a few DPRB; however, the majority of the DPRB isolated to date reside in the  $\beta$  subclass. The fact that the majority of the known (per)chlorate-reducing isolates are either *Dechloromonas* or *Dechlorosoma* species suggests that these groups likely represent the predominant (per)chlorate-reducing bacteria in the environment. This is further supported by the fact that, in almost every environment screened in our studies, members of these two groups were either isolated or their presence was identified by molecular analyses (18, 30-32). Although the Fe(III)-reducing organism, *F. limneticum*, is an anomaly amongst this assemblage of organisms, its environmental significance is still unknown as there is only a single representative of this genus to date (37).

In addition to the treatment of chlorate and perchlorate contamination, previous studies in our lab have demonstrated that the unique metabolic capabilities of (per)chlorate-reducing bacteria can alternatively be used for the treatment of other contaminants including heavy metals, radionuclides (66) and hydrocarbons (29, 30). The only known natural source of (per)chlorate is from mineral deposits in Chile (42, 100) and the presence of (per)chlorate in the environment is the result of anthropogenic contamination over the last one hundred years. It is thus surprising that such a phylogenetically diverse set of organisms should have evolved the ability to grow by dissimilatory (per)chlorate reduction in such a short time frame and suggests that the metabolic capability to grow by the dissimilation of (per)chlorate is the result of a horizontal gene transfer event in the environment. This hypothesis is further supported by the fact that some DPRB are almost identical phenotypically and genotypically to organisms not capable of (per)chlorate reduction. For example, the  $\gamma$  Proteobacterium strain PK is 99.8% similar to *Pseudomonas stutzeri* based on 16S rDNA sequence analysis and phenotypically shares many characteristics of a pseudomonad; yet, strain PK is able to grow by dissimilatory (per)chlorate reduction while *P. stutzeri* cannot (31). The specific genetic mechanisms that confer (per)chlorate reduction and the true role of DPRB in the environment have yet to be identified.

### 5.2.3 Description of *Dechloromonas* and *Dechlorosoma* species.

#### **5.2.3.1 *Dechloromonas***

*Dechloromonas*. De.chlo.ro.mo'nas; L. pref. de from, Gr. adj. chloros green ("chlorine"), Gr. fem. n. monas unit, monad, N.L. fem. n. *Dechloromonas* a dechlorinating monad.

Rod-shaped, gram-negative cells 0.5 by 2  $\mu\text{m}$ , non-sporeforming, non-fermenting, facultative anaerobe. Cells are motile by a single polar flagellum and occur singly or in chains of 2 to 3 cells. A strictly respiring, complete-oxidizer that oxidizes acetate with  $\text{O}_2$ ,  $\text{ClO}_3^-$ ,  $\text{ClO}_4^-$ , or  $\text{NO}_3^-$  serving as alternative electron acceptors. Perchlorate and chlorate are completely reduced to chloride. Cells contain c-type cytochrome(s).

The type species is *Dechloromonas agitata* (sp. nov.), a.gi.ta'ta; L. fem. part. adj. *agitata* agitated, highly active. Cells of *D. agitata* can grow with  $\text{O}_2$ ,  $\text{ClO}_3^-$ , or  $\text{ClO}_4^-$  serving as alternative electron acceptors. Organics used as alternative electron donors include propionate, butyrate, lactate, succinate, yeast extract, fumarate, and malate. The reduced form of the humic substances analogue, 2,6-anthrahydroquinone disulfonate, Fe(II), or sulfide can also serve as alternative electron donors coupled to the reduction of chlorate. Fe(II) is oxidized to insoluble amorphous Fe(III)-oxide while sulfide is oxidized to elemental sulfur. Cells contain c-type cytochrome(s). Optimum growth is observed at 35 °C, pH 7.5, and 1% NaCl with acetate (10 mM) as the electron donor and chlorate (10 mM) as the electron acceptor. The mol% G+C is 63.5.

The type strain of *Dechloromonas agitata*, strain CKBT has been deposited in the American Type Culture Collection under ATCC 700666

#### 5.2.3.2 *Dechlorosoma*

*Dechlorosoma* (gen. nov.), De.chlo.ro.so'ma; L. pref. de from, Gr. adj. chloros green ("chlorine"), Gr. neut. n. soma body, N.L. neut. n. *Dechlorosoma* dechlorinating body.

Cells are gram-negative, facultative anaerobic, non-sporeforming, non-fermentative rods, 1.0 by 0.3  $\mu\text{m}$ . Cells are motile by a single polar flagellum and occur singly or in chains. Simple organic fatty acids are used as electron donors with  $\text{O}_2$ ,  $\text{ClO}_3^-$ ,  $\text{ClO}_4^-$ , or  $\text{NO}_3^-$  as alternative electron acceptors. Organic electron donors are completely oxidized and perchlorate or chlorate are completely reduced to chloride. Cells contain c-type cytochromes.

The type species is *Dechlorosoma suillum* (sp. nov.), su.il'lum; L. neut. adj. suillum pertaining to swine (32). Cells of *D. suillum* strain PST use acetate, propionate, butyrate, casamino acids, lactate and ethanol as alternative electron donors with  $\text{O}_2$ , (per)chlorate or nitrate as the electron acceptor. Nitrate is reduced to  $\text{N}_2$  gas. Optimum growth is observed at 37 °C and pH 6.5 in freshwater media (0 % NaCl). The mol% G+C is 65.8.

The type strain of *Dechlorosoma suillum* strain PST has been deposited in the American Type Culture Collection under ATCC BAA-33.

#### 5.2.3.3 Habitat.

*D. agitata* was obtained from (per)chlorate-reducing enrichments from samples collected from a pulp and paper plants waste pulp slug in Pennsylvania while *D. suillum* was obtained from (per)chlorate-reducing enrichments from samples collected from a primary treatment lagoon of swine waste at the Agricultural Research Facility at Southern Illinois University, Carbondale campus. Acetate (10 mM) was used as the electron donor in the enrichment and isolation cultures for both organisms.

### 5.3. Controlling factors of microbial perchlorate reduction

#### 5.3.1 Effect of oxygen on CD activity and perchlorate reduction by *D. suillum*.

When *D. suillum* was grown anaerobically under an N<sub>2</sub> gas phase with perchlorate as the sole electron acceptor, perchlorate removal was rapid and CD activity was observed (Fig. 5.6a and b). However, if air was introduced into the headspace of the anaerobic culture after 21 hours perchlorate reduction was immediately inhibited despite the continued increase of cell density. Measurement of CD activity indicated that chlorite was readily dismutated by the cells at the 21 hour time point (Fig. 5.6b) when O<sub>2</sub> was introduced to the culture. Subsequent analysis 2 hours and 5 hours after introduction of air indicated that CD was still active although the dismutation rate was significantly lower 5 hours after O<sub>2</sub> introduction. Previous studies have suggested that the presence of oxygen similarly inhibited microbial reduction of chlorate (71). Furthermore, thermodynamic consideration of the respective reductive pathways suggested that the aerobic metabolism of acetate is energetically more favorable than acetate oxidation coupled to perchlorate reduction (32). However, this may be an over simplification as genetic regulation may also play an important role. In support of this, studies with the DPRB, strain GR-1, demonstrated that the CD activity was significantly higher when the cells were grown on perchlorate relative to aerobic cultures suggesting that the CD was induced under anaerobic conditions (93). In addition, immuno-probe studies of the DPRB, *Dechloromonas agitata*, indicated that the CD enzyme was only expressed when the cells were grown on perchlorate (82). Similarly to these findings, CD activity in *D. suillum* was only observed in cells collected from an active culture growing anaerobically with perchlorate as the electron acceptor (data not shown). No CD activity was detectable if the culture was grown aerobically.

#### 5.3.2 Growth and CD expression under anaerobic conditions.

To determine if anoxic conditions alone would induce the production of CD by *D. suillum*, the O<sub>2</sub> content of an actively metabolizing aerobic culture was replaced with N<sub>2</sub> after 4.5 hours incubation during log phase growth. Growth was immediately inhibited relative to an unamended control culture (Fig. 5.7). CD activity was not observed in samples collected at 3.5, 5, and 7 hours incubation from either the aerobic control culture or the culture switched to anaerobic conditions (data not shown) suggesting that anaerobic conditions are not enough to initiate expression of an active CD. In addition, if *D. suillum* was grown aerobically in the presence of 5 mM perchlorate in a chemostat at 100% dissolved oxygen (DO) saturation (~40 mg.L<sup>-1</sup> DO), CD activity was not observed in cells throughout culture growth and perchlorate reduction did not occur (data not shown). Similar results were observed if the culture was grown at lower DO concentrations (70%, 40%, and 5% of saturation) in the presence of 5 mM perchlorate indicating that the presence of perchlorate even at low DO concentrations was not enough to induce perchlorate reduction by *D. suillum*. If, however, the O<sub>2</sub> content of was replaced with N<sub>2</sub> during log phase growth, both perchlorate reduction and CD activity were measurable after an extended period (15 hours) (Fig. 5.7). As observed above, growth was initially inhibited on removal of the O<sub>2</sub> but was recovered after a lag phase of 4 hours (Fig. 5.7). Interestingly, renewed growth was observed prior to detectable perchlorate reduction or CD activity (Fig. 5.7) although this may be a function of the sensitivity of the various measurement techniques utilized.

### 5.3.3 Effect of nitrate on perchlorate reduction and CD expression by *D. suillum*.

Previous studies have demonstrated that all but one of the known DPRB can alternatively utilize nitrate as a suitable electron acceptor. *Dechloromonas agitata* strain CKB is the only DPRB described to date which can not support growth by nitrate respiration (18). If a nitrate-grown culture of *D. suillum* was transferred into fresh anoxic medium, nitrate reduction and growth occurred immediately with no apparent lag phase and CD activity was not observed throughout the incubation (data not shown). In contrast, if a nitrate-grown culture of *D. suillum* was used to inoculate medium containing equimolar amounts of perchlorate and nitrate, all growth and respiration was inhibited for an extended lag period (40 hours) after which nitrate reduction occurred prior to perchlorate reduction (Fig. 5.8a). When a similar experiment was performed with a perchlorate-grown culture inoculated into fresh medium containing equimolar amounts of both nitrate and perchlorate no lag phase was apparent and again nitrate reduction occurred prior to perchlorate reduction (Fig. 5.8b). Regardless of the electron acceptor utilized in the inoculum culture neither CD activity (data not shown) or perchlorate reduction was observed until nitrate was completely consumed (Fig. 5.8a and b).

In contrast to *D. suillum*, the presence of nitrate had little effect on perchlorate reduction by the DPRB *Dechloromonas agitata* strain CKB. As outlined above *D. agitata* cannot use nitrate as an alternative electron acceptor for growth (18). When an active perchlorate-respiring culture of *D. agitata* was transferred into freshly prepared anoxic medium containing both nitrate and perchlorate, the perchlorate was rapidly reduced (Fig. 5.9). Both the rate and extent of perchlorate utilization was less than that observed in the absence of nitrate suggesting that the nitrate was competitively inhibiting perchlorate reduction (Fig. 5.9). Interestingly, although *D. agitata* does not grow by nitrate reduction, the nitrate in the culture medium was concomitantly reduced to nitrite during perchlorate reduction which accumulated in the culture broth (Fig. 5.9) suggesting that the nitrate was being co-reduced by the (per)chlorate reductase. Previous studies performed on the DPRB strain perclace also indicated that nitrate was concomitantly reduced with perchlorate by this DPRB (53). However, in contrast to *D. agitata*, strain perclace could grow by nitrate reduction and nitrite did not accumulate in the culture broth (53).

### 5.3.4 Effect of molybdenum on perchlorate reduction.

Recent molecular studies of the genetic systems associated with perchlorate reduction indicated the presence of a molybdenum-dependent chaperone gene in association with the genes encoding CD and perchlorate reductase in *Dechloromonas aromatica* strain RCB and *Pseudomonas* strain PK (12). Furthermore, the perchlorate reductase enzyme recently purified from the DPRB strain GR-1 contained 1 mole molybdenum per mole of the heterodimeric molecule (59) suggesting that molybdenum may play a functional role in the reduction of perchlorate. In support of this, growth and perchlorate reduction were completely inhibited when an active perchlorate-respiring culture of *D. aromatica* was transferred into medium from which molybdenum was omitted (Fig. 5.10). If the culture was amended with molybdenum after eight hours incubation, growth was immediately recovered (Fig. 5.10) demonstrating a nutritional requirement for molybdenum. Omitting molybdenum from oxic medium had no effect on the aerobic growth of *D. aromatica* (data not shown). Similar inhibition of anaerobic growth and perchlorate reduction was observed with molybdenum depleted cultures of *D. agitata* (data not shown). These results, in combination with the previous genetic and biochemical observations, indicate that molybdenum may be required by all perchlorate-respiring bacteria for the reduction of perchlorate. This may



have important implications regarding bioremediative strategies for perchlorate in contaminated environments as bioavailable molybdenum is quite often a limiting nutrient in many soils. This is especially true in acidic soils where adsorption reduces the availability of molybdenum salts at lower pH values.

#### 5.3.5 Significance.

The results presented here indicate that microbial perchlorate reduction is regulated by the presence of oxygen, molybdate, and to varying extents nitrate. Although, respiration of oxyanions of chlorine was originally associated with the nitrate reduction pathway (39, 46, 48) it is now known that perchlorate reduction is a distinct respiratory pathway with its own unique enzymes (18, 31, 59, 82, 93, 104). The results of the present study indicate that perchlorate reduction is tightly regulated at a genetic level and that the expression of the enzymes associated with the reduction of perchlorate are controlled by the presence of both oxygen and nitrate. Nitrate, (per)chlorate are structurally analogous to each other and may potentially be incorporated into the same enzyme active site as is evidenced by the fact that chlorate can be used as a substrate by various nitrate reductases (6, 44, 96, 132). Thermodynamic considerations of perchlorate reduction and denitrification coupled to acetate oxidation suggest that the energy yield for the reduction of perchlorate ( $\Delta G^\circ = -801 \text{ kJ mol. acetate}^{-1}$ ) is very similar to that of denitrification ( $\Delta G^\circ = -792 \text{ kJ mol. acetate}^{-1}$ ) (32) and the fact that *D. agitata* can simultaneously reduce both perchlorate and nitrate would suggest that nitrate can alternatively be utilized as an analogous substrate by the (per)chlorate reductase enzymes. In support of this the perchlorate reductase purified from the DPRB strain GR-1 could alternatively reduce nitrate as a substrate (59).

Although these compounds are analogous substrates to some reductase enzymes the results presented here also indicate that the genetic regulatory mechanism of *D. suillum* and presumably other DPRB can distinguish between them. The fact that the enzymes associated with perchlorate reduction are genetically regulated by different environmental parameters is interesting, especially in light of recent studies in which it was demonstrated that the CD enzyme is highly conserved amongst the tested DPRB regardless of their phylogenetic affiliation (82).

The results of the current studies have important implications with regards to the treatment of perchlorate-contaminated environments. Previously it was suggested that the inhibitory effects of oxygen and nitrate on in-situ perchlorate reduction were the result of competitive thermodynamics (32). Here we demonstrate that this may have been an over simplification and did not account for genetic regulation of the respective pathways. The results of the present studies demonstrate that the enzymes associated with perchlorate reduction are negatively regulated by dissolved oxygen content, in some cases nitrate, and require molybdenum as a co-factor for activity. These factors may explain the long-term stability of perchlorate in contaminated environments where perchlorate-reducing bacteria are known to be present.

#### **5.4. Isotopic chlorine signature of (per)chlorate and its fractionation during microbial metabolism**

Variations of the stable isotope ratios of many elements have been used for a long time to give valuable information about biogeochemical processes occurring in the environment (9, 64, 81).

Many atoms can exist in two or more forms, chemically identical but differing in mass. The relative abundances of the stable (non-radioactive) isotopes are effectively constant for each element, however, microbial processes are known to make small but significant changes to isotopic compositions (4, 26, 50, 52, 63, 74). In the case of chlorine (Cl) there are relatively few examples of major fractionating processes operating naturally: probably the largest effect being attributed to aqueous diffusion of dissolved chloride in marine porewaters in low permeability rocks (originally ~ 0‰). As a result of the faster diffusion of <sup>35</sup>Cl, dilute brines at the diffusion front have values of ~ -0.9‰ showing relative depletion of <sup>37</sup>Cl, while the residual brine is ~ +1.9‰ (3). However, even larger changes result from chemical manufacturing processes where, for example chlorinated hydrocarbon solvents, products from the common feedstock, natural sodium chloride (~ 0‰), can show a range of values from -3‰ to +4‰ (58). As part of this work we characterized the stable isotopic signature for the chlorine content of (per)chlorate and chlorate in a range of flashpowder and weedkiller samples to explore the use of the technique for characterization of those compounds potentially for forensic purposes. In addition, we also investigated the ability of perchlorate reducing bacteria to fractionate the stable isotope content of perchlorate through reductive dissimilation. It should be noted that isotopic signature characterization might be achieved either by identifying a specific isotopic composition or a range of values that might describe the heterogeneity of a material as shown in the initial work where different grain sizes gave different values. Chlorine isotope values are expressed as the difference between the isotope ratio of the sample and a standard, expressed in parts per thousand (per mil or ‰):

$$\delta^{37}\text{Cl} = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} * 1000$$

where R is the isotope ratio <sup>37</sup>Cl/<sup>35</sup>Cl. The standard is Standard Mean Ocean Chloride (SMOC) which has no measurable variation in composition (Kaufmann et al., 1984a).

#### 5.4.1 Flashpowders.

The results are shown in Table 5.4. Samples showing evidence of air contamination, monitored by measuring mass 28 (nitrogen) are shown in bold italic and are not included in the mean calculations. The results have two features of interest. The variation for replicate analyses is noticeably greater than usual analytical uncertainty. This suggests, but does not prove, sample heterogeneity. However, regardless of the cause, this makes it more difficult to be certain of the other feature of interest in the data, the apparent bimodal distribution of <sup>37</sup>Cl values for the perchlorate. The fact that the variation could be attributed to sample heterogeneity implies there may be some physically detectable within-sample differences. Previous work (Ader et. al., 2001) and the results for weedkiller chlorate in this report show that different grain-sizes may display characteristic isotopic compositions. The bulk sample may manifest this complexity as a greater than expected variation. However, for flashpowder samples which are prepared by mixing finely-ground components, it may not be possible to establish a clear grain size distribution for sub-sample analysis, however, the potential danger of actively grinding the mixed materials may allow original grain sizes or textures to be preserved. The possible bimodal compositional variation might imply two different primary sources of perchlorate used for the manufacture of white, blind and thunderflash in one case; and the amber and red flashpowders in the other. Further speculation is not warranted without better statistical analysis of the existing data and/or

further analyses of similar samples. In addition, there is, as yet, no database of analyses of manufactured crude perchlorate nor any understanding of the effects, if any of refining the material and thus the possible quantification of the extent of inter-batch variation.

#### 5.4.2 Weedkillers.

For the two commercial samples analyzed, the  $^{37}\text{Cl}$  values for chlorate are not significantly different from each other. However, the totality of the results for the all components analyzed shows a richness of characteristic features, which clearly differentiate the two samples. The separation of the samples into three grain-size fractions has proved to be very valuable. Replicate sample preparations showed excellent reproducibility consistent with that expected of the analytical methods used. The first point of note is that the chlorate only occurs in the coarsest size fraction,  $>250\text{ m} < 500\text{ m}$ . The other two size fractions in duplicate preparations, showed no presence of chlorate, in fact the finest size fraction,  $>1\text{ m} < 2\text{ m}$ , showed neither chlorate nor chloride. However, the medium grain and coarse grain size fractions both contained measurable contaminant chloride, the isotopic compositions of which clearly differentiate the two samples. The trend of more positive  $^{37}\text{Cl}$  for chloride for just these two samples would not be very persuasive by itself. However, in conjunction with previous data, which follow a similar trend (Jendrzejewski et al., 2001; Ader et al., 2001), and the fact that the crystallization process would be expected to give such an effect, these results are reassuring. The robustness of these data allows confidence in their use as a characteristic discriminator between the samples. These results imply that the chloride is added intentionally as a component of the mixture and is not the product of degradation of the chlorate. This is excellent support for concept of use of the isotopic composition of the chlorate as a characteristic of the material, which could be changed by degradation. Where there is within-sample heterogeneity it would be usual to validate the totality of the individual results by calculating a weighted average value for the whole sample to be compared with a carefully taken, representative sub-sample of the bulk material. This is not possible in this case since the size fractions taken do not appear to cover all the grain sizes possible. As in the case of the flashpowder samples, there is no database of analyzed samples with which to compare these results and possibly relate them to a specific manufacturer, process or batch.

#### 5.4.3 Microbial chlorine isotope fractionation.

Although several molecular and immunological tools based on unique signature molecules are now available to monitor the microbial populations associated with perchlorate reduction in the environment (12, 13, 82), monitoring of the effectiveness of a bioremediative strategy in field environments is often difficult due to the complex nature of environmental samples. Results can often be tainted by many abiotic factors including adsorption or chemical reactivity of the target contaminant. One potential strategy for overcoming these shortcomings with many compounds is to follow the changes in stable isotope composition of the molecule of interest.

Variations of the stable isotope ratios of many elements have been used for a long time to give valuable information about biogeochemical processes occurring in the environment (9, 64, 81). Many atoms can exist in two or more forms, chemically identical but differing in mass. The relative abundances of the stable (non-radioactive) isotopes are effectively constant for each element, however, microbial processes are known to make small but significant changes to isotopic compositions (4, 26, 50, 52, 63, 74). In the case of chlorine (Cl) there are relatively few

examples of major fractionating processes operating naturally: probably the largest effect being attributed to aqueous diffusion of dissolved chloride in marine porewaters in low permeability rocks (originally  $\sim 0\text{‰}$ ). As a result of the faster diffusion of  $^{35}\text{Cl}$ , dilute brines at the diffusion front have values of  $\sim -0.9\text{‰}$  showing relative depletion of  $^{37}\text{Cl}$ , while the residual brine is  $\sim +1.9\text{‰}$  (3). However, even larger changes result from chemical manufacturing processes where, for example chlorinated hydrocarbon solvents, products from the common feedstock, natural sodium chloride ( $\sim 0\text{‰}$ ), can show a range of values from  $-3\text{‰}$  to  $+4\text{‰}$  (58). Because of its inherently conservative nature, Cl isotope methods have been used and proposed for use as a natural tracer of sources and mixing processes of solutions. Recently, however, studies demonstrated that the chlorine isotope composition of the chlorinated solvents PCE and TCE can be altered during microbial reductive dechlorination and the lighter isotope ( $^{35}\text{Cl}$ ) is preferentially utilized. If perchlorate-reducing bacteria would similarly fractionate the chlorine isotope composition of perchlorate, this would offer a simple yet effective mechanism to monitor the success of a bioremediative strategy for perchlorate contaminated environments.

As part of a study on the metabolic diversity of organisms capable of growth by the anaerobic respiration of perchlorate, we isolated a novel organism, *Dechlorosoma suillum* strain PS, from a swine waste lagoon (1, 31, 76). Physiological characterization revealed that *D. suillum* rapidly reduced perchlorate with acetate as the electron donor and that reduction was dependent on molybdate and was negatively regulated by the presence of either oxygen or nitrate (12, 22, 82). The ability of *D. suillum* to fractionate chlorine isotopes while growing on perchlorate was determined by culturing the organism at  $37\text{ }^{\circ}\text{C}$  in modified basal medium (18) from which the major chloride salts were removed and replaced with equivalent sulfate salts. Culturing was performed with acetate (10 mM) as the sole electron donor and perchlorate (10 mM) as the sole electron acceptor in a 6L automatically controlled (pH, temperature, and dissolved oxygen) batch fermenter (New Brunswick Scientific Co. Inc, Edison, NJ; Model: Bioflow 2000) as previously described (23). A value of pH 7.0 was maintained through the automatic dispensation of anoxic sterile solutions of either 0.5 M  $\text{H}_2\text{SO}_4$  or 1.0 M NaOH as appropriate. The inoculum culture of *D. suillum* was prepared in an identical chloride-free medium except that nitrate (10 mM) replaced perchlorate as the sole electron acceptor. Growth was monitored by microscopic observation and optical density measurements at 600 nm. Perchlorate and chloride concentrations were determined on subsamples collected at regular intervals throughout the growth cycle by ion chromatography analyses as previously described (22). Ion chromatographic analysis of the basal medium components prior to inoculation indicated the presence of a minor chloride contamination (0.2 mM) resulting from the vitamin and mineral stock solutions which was expected as some chloride salts were used in the preparation (18).

Growth and perchlorate reduction by *D. suillum* was rapid and reproducible in this medium (Fig. 5.11a). The initial lag phase was due to the transfer of the nitrate grown culture into medium with perchlorate as the sole electron acceptor as previously observed (23). Ion chromatographic analysis (23) of the culture broth throughout the growth phase revealed that the perchlorate was quantitatively reduced to chloride by *D. suillum* and no potential intermediates such as chlorate or chlorite were observed (Fig. 5.11b). Subsamples were collected throughout the growth phase of the culture for determination of the chlorine isotope composition of the perchlorate. Cells were removed by filtration through sterile  $0.2\text{ }\mu\text{m}$  pore size filters under an  $\text{N}_2\text{-CO}_2\text{-H}_2$  (75%-20%-5%, vol/vol) atmosphere in an anaerobic glove bag (Coy Laboratory Products Inc, MI) and

dispensed into previously prepared, clean, sterile sealed 10 ml serum vials filled with N<sub>2</sub>. Isotopic analysis of the perchlorate and residual chloride contents of the samples was performed as previously described (3). Briefly, chloride was separated from the perchlorate by precipitation as AgCl<sub>2</sub> and the perchlorate content of the samples was then quantitatively reduced to chloride by alkaline fusion-decomposition as previously described (3). As before, stoichiometric conversion of the perchlorate to chloride (> 98% yield) was achieved (data not shown). Chlorine isotope analyses were performed in duplicate on each sample. The precision of a single isotopic analysis was <0.05‰ and the average difference between duplicate analyses was 0.1‰. The results indicated a systematic variation in isotopic composition as the extent of perchlorate reduction to chloride by *D. suillum* proceeded (Fig. 5.12). The relationship between the isotope composition of the residual product, the fraction reacted (f) and the isotopic difference between reagent and product ( $\alpha$ ) was a function of a variant of the Rayleigh Distillation formula,  $R/R_0 = f^{(\alpha-1)}$ , where  $R_0$  is the <sup>37</sup>Cl/<sup>35</sup>Cl of initially present Cl<sup>-</sup> in ClO<sub>4</sub><sup>-</sup>; R is the <sup>37</sup>Cl/<sup>35</sup>Cl of residual ClO<sub>4</sub><sup>-</sup>;  $\alpha$  is the isotopic fractionation factor for the reaction,  $R_{\text{perchlorate}}/R_{\text{chloride}}$ . The equation operates similarly but independently with the relevant chloride isotope data. Perchlorate isotope data from an initial experiment were consistent with a very significant isotopic fractionation factor,  $\alpha = 0.9842 \pm 0.0004$ , equivalent to an isotopic difference, perchlorate-chloride =  $15.8 \pm 0.4\text{‰}$  (mean  $\pm$  standard deviation, n=3) (Fig. 5.12). Because if the low level chloride contamination in the basal medium associated with the use of chloride salts in the trace metal and vitamin stock solutions chloride data were not of sufficient quality to calculate an independent estimate of  $\alpha$ . However, two more-extensive repeat experiments gave a  $\Delta_{\text{perchlorate-chloride}}$  value of  $14.8 \pm 1.3$  from perchlorate chlorine isotopes while chloride gave an independent determination of  $14.8 \pm 0.7\text{‰}$  (both, mean  $\pm$  standard deviation, n=10). Although the isotopic differences from all the experiments are not identical, they are very consistent and are indicative of an exceptional level of fractionation and the robustness of the data.

The extent of this fractionation is much greater than for chlorine isotopic fractionation (< -2.2 ‰) previously observed during microbial reductive dechlorination of TCE and tetrachloroethene (cDCE). Interestingly, although previous studies have demonstrated that microbial fractionation of some stable isotopes, such as sulfur, are a function of the rate of microbial reduction, the results presented here indicate that the microbial fractionation of chlorine by *D. suillum* was constant throughout growth and show no indication of any rate-dependent effects (Fig. 5.12). Similar reduction rate-independent chlorine isotope fractionation was previously observed during the reductive metabolism of trichloroethene (TCE) by halo-respiring organisms utilizing either pyruvate or hydrogen as alternative electron donors.

Intrinsic remediation is the preferred option for remediation of perchlorate-polluted sites (116). The results presented here represent the first demonstration of the microbial fractionation of the chlorine stable isotopes of perchlorate. The discovery of the large isotopic fractionation factors associated with microbial perchlorate reduction allows the measurement of the rate and extent of natural or engineered bioremediation at any site and the discrimination between biotic removal of perchlorate from any possible abiotic mechanisms. Currently little is known about abiotic mechanisms which may result in the chemical reduction or attenuation of perchlorate in the natural environment. As such, the distinction between biological and abiotic mechanisms such as dilution, dispersion or adsorption, during the treatment of a perchlorate-contaminated site is an essential part of monitoring the success of a bioremediation strategy. The use of chlorine

isotopic signature determination throughout a bioremediative process may also be of use for the development and application of appropriate predictive models to identify future movement of the pollutant and its potential impact on sensitive environments. Equally important is the possibility of using the mass and bulk isotopic balances to determine an independent validation of the approach.

## **5.5. Perchlorate contaminated site characterization**

### **5.5.1 San Nicholas Island, CA (SNI)**

#### **5.5.1.1 Geochemistry**

Geochemical analyses were performed on soil and water samples collected from three depths at three different sites on the Naval facility at San Nicolas Island, CA. This facility is currently used for surplus munitions burial and continued weapons testing. The island is inhabited with various military personnel, civilian engineers and scientists. The island itself is comprised of a mixture of sand and soil particles; however, most of the testing grounds are very sandy in composition and the subsurface samples from the contaminated sites had high concentrations of caliche present. Results of geochemical analyses (Table 5.5) revealed that all samples were alkaline with a pH range of 8.0 – 9.0, nitrate concentrations were negligible and sulfate was not present to any appreciable extent (Table 5.5). In contrast, all sampled sites had abundant total iron, ranging from a low of 6.34 mmoles per Kg soil to a high of 55.01 mmoles per Kg soil. The percent ferrous iron content increased with depth at each site, suggesting that the soil was anaerobic (Table 5.5). At the lower depth of the heavily contaminated site (sample H 9), more than 85% of the iron content was in the reduced state.

#### **5.5.1.2 Perchlorate content**

Perchlorate analysis revealed the presence of a significant concentration (27.69  $\mu\text{M}$ ) of perchlorate in the heavily contaminated site at the shallow depth. Perchlorate concentration decreased with depth at both the heavily contaminated and medium contaminated sites (Table 5.5), suggesting that either perchlorate contamination is migrating from the surface soils or perchlorate is being more rapidly removed in the lower depths. Interestingly, a small amount of perchlorate (0.68  $\mu\text{M}$ ) was detected in the shallow soils of the pristine site, demonstrating the pervasiveness of this contaminant at the San Nicholas facility.

#### **5.5.1.3 TEAP determinations**

Terminal Electron Accepting Process (TEAP) determinations were performed on all soil and sediment samples by monitoring the production of  $^{14}\text{CO}_2$  from sample incubations in the presence of ( $^{14}\text{C}$ )-acetate under anoxic conditions. The results of these analyses indicated that the majority of the samples collected were dominated by aerobic microbial populations as production of  $^{14}\text{CO}_2$  from the ( $^{14}\text{C}$ )-acetate was negligible under the anaerobic conditions utilized, regardless of the molybdate concentrations. However, some isolated samples such as soils from the shallow, heavily contaminated (H 3) site showed inhibition of  $^{14}\text{C}$ - $\text{CO}_2$  produced in the presence of 20 mM molybdate, suggesting that these samples do have an active sulfate-reducing population (Fig. 5.13). In contrast however, the lower sediment cores (P 51/2 and P 8) showed no inhibition of  $^{14}\text{CO}_2$  produced in the presence of molybdate suggesting that these samples contained an active Fe(III)-reducing population.

#### **5.5.1.4 MPN counts for perchlorate-reducing bacteria**

Most Probable Number (MPN) enumeration studies performed on all samples from San Nicolas Island, California revealed the presence of significant populations of acetate-oxidizing perchlorate-reducing bacteria at the medium contaminated and pristine sites only. Unexpectedly, no perchlorate-reducing bacteria were detected at the heavily contaminated site (Table 5.6). The population ranged from a high of  $2.4 \times 10^5$  cells/gram DPRB at the lower depths of the pristine site (P 51/2 and P 8), (Table 5.6) to a low of  $7.49 \times 10^4$  cells/gram DPRB at the explosive ordinance disposal sediments, (M 3 and M 6).

#### **5.5.1.5 PCR of gDNA isolated from highest dilution MPN tube**

Previous studies performed in the Coates lab identified three new environmentally dominant genera of perchlorate-reducing bacteria, *Dechlorospirillum* (strain WD), *Dechloromonas* (strains CKB and RCB), and *Dechlorosoma* (strain PS), which represent the most commonly isolated perchlorate-reducing bacteria. Analyses of the complete 16S rDNA sequence of these three genera resulted in the identification of signature nucleotide sequences from which primers specific for each genus were designed. These primers bind with various degrees of efficiency to close phylogenetic relatives of each type strain but distinguish between these and their non-perchlorate-reducing relatives. As such, these primers represent useful tools in determining the ubiquity of these respective species of perchlorate-reducing bacteria in environmental samples. Total genomic DNA was extracted from the highest positive dilution tubes of the MPN series from each soil sample and amplified using the specific primers to determine the presence of WD-type, CKB-type, RCB-type, or PS-type organisms. Agarose gels were used to visualize PCR products and these products were compared to amplifiable PCR products of known origin (Fig. 5.14). The results indicated that all of the highest dilution MPN tubes that had growth from the SNI facility were negative for the four different (WD-, CKB-, RCB-, and PS-type) primer sets that detect the most commonly isolated perchlorate reducers (Table 5.7). Similarly, 16S rDNA amplifications of DNA directly extracted from the original soil samples collected from each site also indicated that PCR products were not formed despite the fact that PCR products were obtained using universal bacterial 16S primers (Table 5.8). These results suggest that although perchlorate-reducing populations are prevalent in the soils collected from San Nicholas Island, the population was not comprised of WD-, CKB-, RCB-, or PS-type organisms.

### **5.5.2 Longhorn Army Ammunition Plant, Longhorn (LHAAP), TX**

#### **5.5.2.1 Geochemistry**

Geochemical analyses of these samples revealed that in contrast to San Nicholas Island, the LHAAP sites were acidic, with a pH range of 4.2 – 6.6 (Table 5.9). The lowest pH was observed in surface soil samples collected from site 25 while the highest pH was observed in sediments collected from site 16 (Table 5.9). Similarly to the SNI samples nitrate levels in all samples from LHAAP were negligible. Sulfate levels varied considerably from sample to sample and ranged from a low of 0.15 mmol per Kg sample in surface soils collected from site 25 to a high of 29.61 mmol per Kg sample in sediment collected from site 16 (Table 5.9). Total iron concentrations showed similar variability across the selected samples, ranging from 1.25 to 32.26 mmol per Kg sample, and were predominantly in the oxidized ferric form (Table 5.9).

#### **5.5.2.2 Perchlorate content**

Perchlorate analysis revealed the presence of significant concentrations (792.5  $\mu\text{M}$ ) of perchlorate in the samples collected from LHAAP. These concentrations ranged from a low of 2.21  $\mu\text{M}$  in groundwater collected from site 16 to a high of 792.5  $\mu\text{M}$  in sediment collected from site 25 (Table 5.9). Interestingly the perchlorate concentrations at site 25 were highest in the deepest samples 12 – 16 ft below surface, suggesting that the perchlorate is migrating downwards from the surface with water from the mixing facility.

#### **5.5.2.3 TEAP determinations**

TEAP determinations were performed on all soil and sediment samples collected from LHAAP. Water samples were not used for TEAP determination assays. TEAP analyses indicated that ( $^{14}\text{C}$ )-acetate was slowly oxidized to  $^{14}\text{CO}_2$  under anaerobic conditions by the microbial populations in both the surface soils and sediment samples from site 25, suggesting that the indigenous populations were aerobic (Fig. 5.15). The presence of molybdate inhibited  $^{14}\text{CO}_2$  production by the microbial population in the surface soil samples suggesting that under the conditions of incubation sulfate-reducing bacteria were predominant. In contrast, no molybdate inhibition was observed in the sediment samples (Fig. 5.15).

In contrast to site 25, the microbial population in sediment collected from site 16 showed rapid utilization of the ( $^{14}\text{C}$ )-acetate and the catabolism had gone to completion prior to the first sampling point 10 hours after acetate addition (Fig. 5.15), indicating the presence of an active anaerobic microbial population. The addition of molybdate caused a significant inhibition of  $^{14}\text{CO}_2$  production, indicating that sulfate-reducing bacteria dominated this population, which is consistent with the geochemical data obtained.

#### **5.5.2.4 MPN counts for perchlorate-reducing bacteria**

MPN enumeration studies performed at the environmental pH revealed the presence of significant numbers of acetate-oxidizing, perchlorate-reducing bacteria in all samples collected from LHAAP ranging from a low of  $1.12 \times 10^3$  to a high of  $2.4 \times 10^6$  cells/gram sample (Table 5.10). Interestingly, the highest numbers were associated with sediment and groundwater collected from site 16 which had the lowest level of contamination.

#### **5.5.2.5 PCR of gDNA isolated from highest dilution MPN tubes**

MPN-PCR analysis of the microbial perchlorate-reducing population in the highest positive dilution tubes of the MPN series for LHAAP indicated that the dominant acetate-oxidizing perchlorate-reducing bacteria in these samples were not members of the WD, CKB, RCB, or PS subgroups (Table 5.11). Similar negative results were obtained with genus-specific amplifications of DNA directly extracted from the original soil, sediment, and water samples collected from each site (Table 5.12). These results suggest that, similarly to San Nicholas Island, the perchlorate-reducing population at LHAAP was not comprised of WD-, CKB-, RCB-, or PS-type organisms although perchlorate-reducing populations are prevalent in these samples. This is probably because the pH of this environment is lower than the optimum pH for growth of these organisms.



#### **5.5.2.6 Activity assay for Longhorn, Texas Soils**

Interestingly, activity assays indicated that the indigenous perchlorate-reducing populations at Longhorn, Texas did not reduce perchlorate even in the presence of acetate under anoxic conditions. As seen in Figure 5.16, site 16 soils amended with acetate showed no perchlorate reduction over several days. Sites 25C and 25G also shared the same trend as in site 16. The fact that MPN studies indicated that these organisms are abundant in the samples and grow quite readily in selective media used in the MPN tubes suggests that some component or limiting nutrient in the environment is inhibiting their activity.

#### **5.5.3 Isolation of strains LT-1 and AH**

After at least one month of incubation, the highest dilution with apparent growth from each MPN series was transferred (10% inoculum) into fresh basal medium of an appropriate pH containing 5 mM each of acetate and perchlorate as the sole electron donor and acceptor respectively. Growth was detected by an increase in optical density of the culture and by direct microscopic examination. Cultures that continued to grow after at least three consecutive transfers were put into agar shake tubes for isolation. Small colonies of perchlorate-reducing bacteria developed at various rates in the shake tubes after 1 – 2 weeks. Colonies were distributed evenly in the higher dilution shake tubes ( $10^{-7}$ ) and individual colonies were picked for isolation by placing the colonies in fresh basal medium of an appropriate pH with 5 mM acetate and 5 mM perchlorate serving as the electron donor and acceptor, respectively. Several isolates were obtained from a variety of pHs, and two were chosen to be the focus of this thesis. They include: strain LT-1, isolated from a pH of 6.6; and, strain AH, isolated from a pH of 4.5, which is the lowest pH at which microbial perchlorate reduction has been observed to date.

#### **5.5.3.1 Cell and colony morphology**

Strains LT-1 and AH are complete-oxidizing, non-fermentative, gram-negative, facultative anaerobes. Strain LT-1 is a motile rod, approximately 0.28 microns in width by 0.8 microns in length (Fig. 5.17). Strain AH is a highly motile, curved rod-shaped bacterium, approximately 0.37 microns in width by 1 to 1.5 microns in length (Fig. 5.18). Both strains appear identical when grown on plates. Spores were not visible in wet-mounts by phase contrast microscopy of either strain and growth was not observed in fresh acetate-perchlorate medium after pasteurization at 80 °C for 3 minutes. When grown on anaerobic plates with acetate as the electron donor and perchlorate as the electron acceptor, colonies were small (1 - 2 mm diam.), smooth, and pink in color.

#### **5.5.3.2 Optimal growth conditions**

Phenotypic characterization revealed that both organisms were freshwater acid-tolerant organisms. Strain LT-1 grew over a pH range of 5.6 to 7.8 with optimum growth being observed at pH 6.6 (Fig. 5.19). Strain AH grew over a broader pH range of 4.5 to 7.0 and grew optimally at pH 5.5 (Fig. 5.20). In addition, strain LT-1 grew over a temperature range of 4° to 37°C, and a salinity range of 0 to 1% sodium chloride. Optimum growth was observed to be pH 6.6, 25°C, and 0% NaCl (Fig. 10). Strain AH grew over a temperature range of 16° to 37°C, and a salinity range of 0 to 1% NaCl. Optimum growth was observed to be pH 5.5, 30°C, and 0% NaCl (Fig. 5.20).

#### **5.5.3.3 Alternative electron donors**

Strain LT-1 and strain AH grew anaerobically in basal medium with acetate as the electron donor and sole carbon source and perchlorate as the terminal electron acceptor. In addition to acetate, strain LT-1 and strain AH were able to grow by perchlorate reduction utilizing alternative simple organic compounds, such as propionate, fumarate, succinate, malate, pyruvate, as well as yeast extract, and casamino acids. In addition, strain LT-1 was capable of growing by perchlorate reduction using volatile fatty acids such as butyrate, valerate, isovalerate, and hexanoate, as well as hydrogen gas. Strain AH was able to utilize both ethanol and lactate as electron donors. Strains LT-1 and AH were unable to grow on any of the sugars tested either by perchlorate reduction or fermentatively, or when placed in basal medium containing yeast extract (1gm/l), casamino acids (1gm/l), and glucose (10 mM), and neither bacterium were capable of utilizing any of the hydrocarbons tested (Table 5.13).

#### **5.5.3.4 Alternative electron acceptors**

Both strain LT-1 and strain AH had a limited number of alternative electron acceptors. In addition to perchlorate, these strains could only utilize chlorate, nitrate, or oxygen when grown in basal medium with acetate serving as the electron donor. Other electron acceptors commonly found in anaerobic environments such as sulfate or ferric iron were not used by either strain.

#### **5.5.3.5 Chlorite dismutase activities**

Similarly to all other tested (per)chlorate-reducing bacteria, strain LT-1 and strain AH could dismutate chlorite into chloride and O<sub>2</sub>. No chlorite dismutation occurred in the absence of cells or if the cells were heat-killed indicating that chlorite dismutation was enzymatically mediated and was not the result of an abiotic reaction.

#### **5.5.3.6 Phylogenetic analysis and primer specificity**

Phylogenetic analysis based on 16S rDNA sequence was performed by Dr. Laurie A. Achenbach for both strains LT-1 and AH. This analysis revealed that strain LT-1 is a member of the  $\beta$ -subclass of the Proteobacteria. Unexpectedly, strain LT-1 was a deep-branching member of the *Dechloromonas* genus. As such, it does not contain the signature nucleotide sequence characteristic of this genus. Thus, the two primer sets that currently detect the *Dechloromonas* genus (CKB- and RCB-type) were not compatible with the 16S rDNA sequence of strain LT-1. Similar phylogenetic analysis of strain AH indicated that it was also a member of the  $\beta$ -subclass of the Proteobacteria. Strain AH was a deep-branching member of the *Dechlorosoma* genus. Strain AH also lacked the signature nucleotide sequence against which the PS-type specific primer sequences targeted. Bootstrap analysis on 100 replicates highlighted the robust nature of the phylogenetic tree topologies. All relevant nodes displayed 100% support with the exception of the node that involved strain LT-1 in the *Dechloromonas* genus (85%; Fig. 5.21).

#### **5.5.4 Environmental pH and its effect on microbial populations**

In situ pH levels vary according to the geochemical characteristics of the environment and these characteristics can change drastically as a result of seasonal events like acid rainfall. These changes in the pH of the environment can select different microbial populations: for example, archeal versus bacterial or gram-positive versus gram-negative may be selected. Also, changes in environmental pH can indirectly affect the indigenous microbial populations by either

precipitating or solubilizing various compounds like humic substances and metals like copper, which at low pH is toxic to some microorganisms. Previous studies have demonstrated that the perchlorate reducers of the class  $\beta$ -Proteobacteria represent two novel genera, the *Dechloromonas* species and the *Dechlorosoma* species (28). The *Dechloromonas* genus is comprised of two subgroups, the CKB-type and RCB-type, while the *Dechlorosoma* genus is comprised of a single monophyletic group (PS-type). Members of these three groups are known to be ubiquitous (28) and have been identified and isolated from nearly all environments screened, including pristine and contaminated fields, ex-situ bioreactors treating perchlorate-contaminated wastes (28), and even in soil and lake samples collected from Antarctica (L.A. Achenbach, unpublished data). As such, these two genera are considered to represent the dominant perchlorate-reducing bacteria in the environment (28). Pure culture studies have demonstrated that members of these genera can grow over a broad range of environmental conditions; however, they generally grow optimally at pH values near neutrality in freshwater environments (28).

The results of the present study demonstrate that although the perchlorate-reducing population of a soil or sediment is significantly affected by the pH of the environment, the population is still composed of members of the *Dechloromonas* and *Dechlorosoma* genera and this further supports the hypothesis that these genera represent the dominant perchlorate-reducing bacteria in the environment (28). In addition, this study also provides the first examples of acidotolerant, perchlorate-reducing bacteria in pure culture.

#### 5.5.5 Bioremediative potential of perchlorate in adverse pH environments

The results presented here demonstrate the potential for microbial perchlorate reduction at adverse pH environments. Most probable number enumeration studies revealed that, similarly to other diverse environments (31), acetate-oxidizing, perchlorate-reducing bacteria were prevalent in samples collected from both alkaline and acidic soils and sediments. In contrast to the findings of previous studies (31), however, biogeochemical analyses of alkaline samples collected from San Nicholas Island Naval facility (SNI) and acidic samples collected from the Longhorn Army Ammunition Plant (LHAAP) revealed that, although both sites contained a significant population of perchlorate-reducing bacteria, these populations were not comprised of perchlorate-reducing bacteria commonly found in neutral pH freshwater environments. PCR amplification reactions using 16S rDNA primers targeted against signature nucleotide sequences of both the *Dechlorosoma* and *Dechloromonas* genera did not yield any reaction products, suggesting that these organisms are not represented to any significant extent in these environments. However, the isolation of strains LT-1 and AH from the LHAAP site could in part explain these results. These organisms were directly isolated from the highest positive dilution of the MPN series indicating that they represent the dominant perchlorate-reducing bacteria in these samples. The phylogenetic analysis of these strains placed strain LT-1 in the *Dechloromonas* genus and strain AH in the *Dechlorosoma* genus. However, both strains represented deep branches within these genera and do not contain one of the two characteristic signature nucleotide sequences of both the *Dechlorosoma* and *Dechloromonas* against which the specific primer sets were targeted. This was confirmed by the negative results obtained when performing PCR amplification reactions with the respective specific primer sets and DNA extracted from pure cultures of the two isolates.

Interestingly, although perchlorate-reducing bacteria were found at both facilities, the population size was not directly correlated to perchlorate concentration; in samples originating from the highly contaminated zone collected at San Nicholas Island, no acetate-oxidizing, perchlorate reducers were detected using the MPN technique. These results suggest that active perchlorate respiration may not be occurring at any of the sites sampled. This is further supported by the results of the TEAP analyses, which revealed that most of the samples were dominated by aerobic microbial populations, suggesting that the sites were not limiting for dissolved oxygen. Previous studies performed with pure cultures of perchlorate-reducing bacteria demonstrated that the activity of perchlorate-reducing bacteria was negatively regulated by the presence of oxygen (22, 84). Dissolved oxygen concentrations of less than 2 mg L<sup>-1</sup> were enough to inhibit perchlorate reduction (22). Similarly, nitrate also inhibited perchlorate reduction to varying extents, depending on the organism tested (22). Nitrate inhibition was unlikely to be significant at either the SNI or LHAAP site as nitrate concentrations at both sites were negligible.

Strains AH and LT-1 represent the first examples of acidotolerant, perchlorate-reducing bacteria in pure culture. Strain LT-1 grew optimally at a pH of 6.6, while strain AH grew optimally at a lower pH of 5.5, although both were capable of growing at significantly lower pH values. Previous phenotypic characterizations of perchlorate-reducing bacteria suggest that these organisms in general grow optimally at circumneutral pH (28). For example, *Dechlorosoma suillum* grew optimally at pH 7.2 (76) while *Dechloromonas agitata* grew optimally at pH 7.5 (18).

Both strains AH and LT-1 were members of the  $\beta$ -subclass of the Proteobacteria, closely related to the phototrophic *Rhodocyclus* species. In contrast to *Rhodocyclus* species, strains AH and LT-1 are motile, non-fermentative rods that do not contain bacteriochlorophyll and cannot grow photosynthetically. Strain LT-1 is a deep-branching member of the previously described *Dechloromonas* genus, while strain AH is a deep-branching member of the previously described *Dechlorosoma* genus. Members of both of these genera are known for the ability to grow by perchlorate reduction (1, 28). Strains AH and LT-1 are phenotypically similar to the other known perchlorate-reducing bacteria in these genera; both have the ability to couple growth to the mineralization of acetate with (per)chlorate serving as alternative electron acceptors. (Per)chlorate is completely reduced to chloride. Only one member of the *Dechloromonas* genus, *Dechloromonas* strain JJ, is known that cannot grow by this metabolism (27).

#### 5.5.6 Comparison of chlorite dismutase of strains AH and LT-1 with other known DPRB

Similar to all other (per)chlorate-reducing bacteria tested, strains AH and LT-1 contain chlorite dismutase activity. The transformation of chlorite by strains AH and LT-1, like that of other (per)chlorate reducers, is not dependent on the presence of acetate. The quantitative dismutation of chlorite into chloride and O<sub>2</sub> is now known to be a central step in the reductive pathway of (per)chlorate that is common to all (per)chlorate-reducing bacteria (31). Chlorite dismutation by (per)chlorate-reducing bacteria is mediated by a highly conserved single enzyme, chlorite dismutase (CD) (28, 31, 84). Studies with washed, whole-cell suspensions demonstrated that the CD was highly specific for chlorite and none of a broad range of alternative analogous anions tested served as substrates for dismutation (18). The CD purified from *Dechloromonas agitata* was a homotetramer with a molecular mass of 120 kDa and a specific activity of 1,928  $\mu$ mol chlorite dismutated per mg of protein per minute (31). This is similar to the molecular mass and

specific activity observed for the CD previously purified from the (per)chlorate-reducer strain GR-1 (123).

Phenotypic studies with the (per)chlorate-reducers *Dechloromonas agitata* and *Dechlorosoma suillum* indicated that CD activity is only present when the organisms are grown anaerobically on perchlorate or chlorate and expression of the CD is negatively regulated by oxygen and nitrate (22). Furthermore, studies with an immunoprobe specific for purified CD from *Dechloromonas agitata* strain CKB indicated that the CD is present on the outer membrane of all (per)chlorate-reducing bacteria including strain LT-1 (84) and therefore would be susceptible to external pH levels. Those studies also showed that CD is a highly conserved enzyme among these organisms, regardless of their phylogenetic affiliation (84).

Similar to all other members of the *Dechloromonas* and *Dechlorosoma* genera, strains AH and LT-1 couple growth to the complete oxidation of acetate and other simple organic acids (28). Unlike *Dechloromonas aromatica* (27), strain LT-1 did not oxidize aromatic hydrocarbons such as benzene or toluene. Strains LT-1 and AH did not utilize any carbohydrates as alternative carbon and energy sources.

#### 5.5.7 Description of strain AH

*Dechlorosoma acidophilus* (sp. nov.), De.chlo.ro.so.ma; De. L. pref. from; chloros Gr. adj. green (chlorine); soma Gr. n. body; acidus L. adj. sour (acid); philus L. v. loving.

Curved rod-shaped, gram-negative cells, 0.28 by 0.8  $\mu\text{m}$ , non-spore-forming, non-fermenting, facultative anaerobe. Cells are motile and occur singly in liquid medium. A complete oxidizer of acetate that is capable of utilizing  $\text{O}_2$ ,  $\text{ClO}_3^-$ ,  $\text{ClO}_4^-$ , or  $\text{NO}_3^-$  as alternative electron acceptors. Compounds used as alternative electron donors included: organic acids (propionate, lactate, succinate, fumarate, pyruvate, and malate); casamino acids; yeast extract; and ethanol. Optimum growth was observed to be pH 5.5, 30°C, and 0% NaCl with acetate (10 mM) as the electron donor and perchlorate (10 mM) as the electron acceptor.

*Dechlorosoma acidophilus* strain AH was obtained from perchlorate-reducing, acetate-oxidizing MPN tubes inoculated with samples collected from the Longhorn Army Ammunition Plant in Texas. Acetate (5 mM) and perchlorate (5 mM) were used as the sole electron donor and acceptor respectively in both the MPN set and isolation cultures.

#### 5.5.8 Description of strain LT-1

*Dechloromonas hydrogenophilus* (sp. nov.), De.chlo.ro.mo.nas De. L. pref. from; chloros Gr. adj. green (chlorine); monas Gr. n. unit.

Rod-shaped, gram-negative cells, 0.28 by 0.8  $\mu\text{m}$ , non-spore-forming, non-fermenting, facultative anaerobe. Cells are motile and occur singly in liquid medium. A complete oxidizer of acetate that is capable of utilizing  $\text{O}_2$ ,  $\text{ClO}_3^-$ ,  $\text{ClO}_4^-$ , or  $\text{NO}_3^-$  as alternative electron acceptors. Compounds used as alternative electron donors included: organic acids (propionate, succinate, fumarate, pyruvate, and malate); volatile fatty acids (palmitate, butyrate, valerate, isovalerate, and hexanoic acid); casamino acids; yeast extract; and  $\text{H}_2$ . Optimum growth was observed to be

pH 6.6, 25°C, and 0% NaCl with acetate (10 mM) as the electron donor and perchlorate (10 mM) as the electron acceptor.

*Dechloromonas hydrogenophilus* strain LT-1 was obtained from perchlorate-reducing, acetate-oxidizing MPN tubes inoculated with samples collected from the Longhorn Army Ammunition Plant in Texas. Acetate (5 mM) and perchlorate (5 mM) were used as the sole electron donor and acceptor respectively in both the MPN set and isolation cultures.

## **5.6 Immunoprobe for perchlorate reducing bacteria**

The SERDP funded studies in our lab have demonstrated the ubiquity and diversity of microorganisms which couple growth to the reduction of chlorate or perchlorate ((per)chlorate) under anaerobic conditions. We identified two taxonomic groups, the *Dechloromonas* and the *Dechlorosoma* groups, which represent the dominant (per)chlorate-reducing bacteria (DPRB) in the environment. As part of these studies we demonstrated that chlorite dismutation is a central step in the reductive pathway of (per)chlorate that is common to all DPRB which is mediated by the enzyme, chlorite dismutase (CD). Initial studies on CD suggested that this enzyme is highly conserved amongst the DPRB, regardless of their phylogenetic affiliation. As such this enzyme makes an ideal target for a probe specific for these organisms. Polyclonal antibodies were commercially raised against the purified CD from the DPRB *Dechloromonas agitata* strain CKB. The obtained antisera were deproteinized by ammonium sulfate precipitation and the antigen binding activity was assessed using dot-blot analysis of a serial dilution of the antisera. The titer values obtained with purified CD indicated that the antisera had a high affinity for the CD enzyme and activity was observed in dilutions as low as  $1 \times 10^{-6}$  of the original antisera. The antisera was active against both cell lysates and whole cells of *D. agitata*, but only if the cells were grown anaerobically with (per)chlorate. No response was obtained with aerobically grown cultures. In addition to *D. agitata*, dot-blot analysis employed with both whole cell suspensions and cell lysates of several diverse DPRB representing the alpha, beta, and gamma subclasses of the Proteobacteria tested positive regardless of phylogenetic affiliation. Interestingly, the dot-blot response obtained for each of the DPRB cell lysates was different suggesting that there may be some differences in the antigenic sites of the CD protein produced in these organisms. In general, no reactions were observed with cells or cell lysates of the closely related organisms to the DPRB which could not grow by (per)chlorate reduction.

These studies have resulted in the development of a highly specific and sensitive immuno-probe based on the commonality of the chlorite dismutase enzyme in DPRB which can be used to assess dissimilatory (per)chlorate-reducing populations in environmental samples regardless of their phylogenetic affiliations.

### **5.6.1 Sensitivity and specificity.**

The commercially raised antiserum was partially purified by salt precipitation of serum proteins using ammonium sulfate cuts prior to determination of antigen binding activity. Dot-blot analysis of a serial dilution of the partially purified immunoglobulin (IgG) against the purified CD ( $5 \mu\text{g } \mu\text{L}^{-1}$ ) indicated that the IgG had a high affinity for CD and activity was still observed at dilutions as low as  $1 \times 10^{-6}$  of the original IgG (Fig. 5.22). No response was observed in similar experiments performed with bovine serum albumin (BSA) ( $5 \mu\text{g } \mu\text{L}^{-1}$ ) in place of the

purified CD (data not shown). Western blot analysis of cell lysates of *D. agitata* indicated that the IgG only reacted with a single protein band in the cell lysate (Fig. 2). No cross reactivity of the IgG was observed with cell lysates of *E. coli* (Fig. 5.23). The fact that the IgG reacted with the denatured protein is indicative that the antigenic site of the IgG is located in the subunits and does not need the native homotetramer structure. Comparison of the reactive protein band with the molecular mass marker indicated a molecular mass of 32 kDa (Fig. 5.23). This is similar to the migration of the CD previously observed in the cell lysates (31). Interestingly, the purified CD migrated slightly further than the 32 kDa band of the lysed cell preparations (Fig. 5.23). In contrast, previous results of SDS-PAGE electrophoresis studies performed with freshly purified *D. agitata* CD demonstrated that its mobility was identical to the 32 kDa band observed in the lysed cell preparation (31). The difference in the observed mobility is likely the result of the extended storage (18 months) of the purified CD at  $-20^{\circ}\text{C}$  prior to use which may have resulted in a slight alteration of the protein structure and subsequent SDS-PAGE mobility pattern. In support of this, activity determination of the stored CD indicated that the specific activity had decreased to 569  $\mu\text{mol}$  chlorite dismutated per mg of protein per minute which is only 30% of the original activity (31).

#### 5.6.2 Reaction with other DPRB.

Microbial dissimilatory (per)chlorate reduction is a phylogenetically diverse metabolism and microorganisms with this capability have been placed in four of the five subclasses of the Proteobacteria (31). As such the application of specific molecular probes based on signature nucleotides in the 16S ribosomal DNA sequence are of limited use especially in light of the fact that 16S rDNA sequence analysis indicates that several DPRB are phylogenetically identical to non-(per)chlorate-reducing organisms (2). To test the universal applicability of the IgG, cell lysates of several phylogenetically distinct DPRB representing the alpha, beta, and gamma subclasses of the Proteobacteria including species of *Dechloromonas*, *Dechlorosoma*, *Dechlorospirillum*, *Dechloromarinus*, *Ideonella*, and *Pseudomonas* genera were screened. In addition, cell lysates of closely related organisms to the DPRB which do not grow by dissimilatory (per)chlorate reduction were also investigated. All the DPRB cell lysates tested positive regardless of phylogenetic affiliation (Fig. 5.24). Interestingly, the response obtained for each of the DPRB cell lysates was slightly different (based on dot-blot intensity) suggesting that there may be some minor differences in the CD protein produced in these organisms (Fig. 5.24). Unsurprisingly, cell lysates of the *Dechloromonas* species, especially, *D. agitata* from which the CD was purified showed the most reactivity. No reaction was observed with *E. coli* or with *Pseudomonas stutzeri* a phylogenetically identical species of the DPRB *Pseudomonas* strain PK based on 16S rDNA sequence analysis which cannot grow by (per)chlorate reduction (31) (Fig. 5.24). In contrast, a slight positive reaction was observed in the dot-blot for the phototrophic beta Proteobacterium *Rhodocyclus tenuis* which is the closest non-(per)chlorate-reducing relative of the *Dechloromonas* genus (Fig. 5.24) (1, 18). However, if the dot-blot procedure was repeated on this organism in the absence of the CD-specific IgG, the HRP labeled goat-antirabbit IgG, and the chemiluminescence substrate a similar positive reaction was observed (Fig. 5.24). This suggests that the reaction was not a result of cross reactivity of the IgG, but was rather a reaction between the luminescent phototrophic centers of *R. tenuis* and the photographic film used.

### 5.6.3 Reaction with whole cells.

Interestingly, the IgG also reacted with whole cell preparations of the individual DPRB and not with the negative controls (Fig. 5.24). In general the reaction with the whole cells was less intensive than that observed with the cell lysates. Previous observations made in our laboratory have indicated that DPRB rapidly lyse in the absence of a suitable electron donor or acceptor (30, 65). In order to determine whether this reaction was the result of reaction of the IgG with CD released as a result of autolysis of the cells during the dot-blot procedure immuno-fluorescent micrographs were prepared from dry mounts of *D. agitata* using fluorescein-labeled secondary goat anti-rabbit antibody. Phase-contrast microscopy of the dry mounts indicated that the cells of *D. agitata* were in good condition with little cell lysis (Fig 5.25). Immuno-fluorescent microscopy revealed that the IgG readily reacted with the whole cells and clearly obviated the intact cell structure (Fig. 5.25). Similar studies performed with *E. coli* showed no fluorescence of the whole cells even after extended staining periods. The fact that the IgG reacted with the whole cells suggests that the CD may be present in the cell outer membrane where it is accessible to the IgG. In support of this, previous studies have demonstrated that the chlorite dismutase activity was associated with both the cell membrane and soluble fractions of a lysed-cell preparation of *D. agitata* when prepared using a French press (18, 31). This result suggested that the CD was loosely bound to the membrane and was sheared off by the French press procedure. In contrast, however, the chlorite dismutase activity of the DPRB, strain GR-1, was located exclusively in the soluble fraction (123).

### 5.6.4 Functional response.

As the CD is a central enzyme in the pathway involved in the dissimilatory reduction of (per)chlorate, the CD-specific IgG may potentially be applied to determining the metabolic state of DPRB. Dot-blot analysis indicated that the CD-specific IgG was active only against cells of *D. agitata* grown with either perchlorate or chlorate as alternative electron acceptors (Fig. 5.26). No reaction was observed when *D. agitata* was grown aerobically (Fig. 5.26). Previous kinetics studies by our lab on DPRB indicated that the enzymes involved in perchlorate reduction are induced only under anaerobic conditions in the presence of (per)chlorate (J.D. Coates, unpublished data). As part of these studies we demonstrated that the CD activity was present only when the organisms were actively growing by (per)chlorate reduction. CD activity was not observed if the cells were growing aerobically or with nitrate as an alternative electron acceptor. This implies that the CD-specific IgG will only bind to DPRB which are actively metabolizing (per)chlorate as it is only under these conditions that they produce an active CD. We could not test the reactivity of the CD-specific IgG with nitrate grown cells of *D. agitata* strain CKB as this organism is one of the few DPRB that cannot grow by nitrate reduction (18).

### 5.6.5 Significance.

The present study resulted in the development of an immuno-probe which has a high affinity for chlorite dismutase, a central enzyme involved in the dissimilatory reduction of (per)chlorate by (per)chlorate reducing bacteria. Previous studies indicated that this enzyme is unique to DPRB and phylogenetically close relatives of the known DPRB which cannot grow by reduction of (per)chlorate are incapable of the dismutation of chlorite (2, 18, 31). In support of this, the developed immuno-probe is specific to DPRB and cross reactivity with non-DPRB was not observed, regardless of their phylogenetic similarity. The probe was reactive with both cell lysates and whole cells of all DPRB tested but only when they were actively metabolizing



(per)chlorate. Partiality based on phylogenetic affiliation was not observed indicating that this probe has potential application for monitoring mixed (per)chlorate-reducing populations in environmental samples.

### **5.7. Identification, characterization, and classification of genes encoding perchlorate reductase**

The reduction of perchlorate to chlorite, the first enzymatic step in bacterial reduction of perchlorate, is catalyzed by perchlorate reductase. The genes encoding perchlorate reductase (*pcrABCD*) were characterized from two *Dechloromonas* species. Sequence analysis of the *pcrAB* ORFs showed similarity to  $\alpha$ - and  $\beta$ -subunits of archaeal nitrate reductases and bacterial selenate reductase, dimethyl sulphide dehydrogenase, ethylbenzene dehydrogenase, and chlorate reductase, all members of the dimethylsulfoxide reductase (DMSO) family. The *pcrC* gene showed similarity to a *c*-type cytochrome, while the *pcrD* gene showed similarity to molybdenum chaperone proteins of the aforementioned enzymes. Expression analysis of the *pcrA* gene from *Dechloromonas agitata* indicated transcription only under anaerobic (per)chlorate reducing conditions. The presence of oxygen completely inhibited *pcrA* expression regardless of the presence of perchlorate, chlorate, or nitrate. Deletion of the *pcrA* gene in *Dechloromonas aromatica* abolished growth in both perchlorate and chlorate, but not nitrate, indicating the functional role of the *pcrABCD* genes in perchlorate reduction and not nitrate reduction. Phylogenetic analysis of the PcrA and other  $\alpha$ -subunits of the DMSO family indicated that the perchlorate reductase forms a monophyletic group separate from the chlorate reductase of *Ideonella dechloratans*. The distinct difference between the perchlorate and chlorate reductase was further supported by hybridization analysis of gDNAs from perchlorate- and chlorate-reducing strains using the *pcrA* gene as a probe.

#### 5.7.1 Identification of *pcrABCD* genes.

In the course of characterizing the chlorite dismutase (*cld*) gene (11), we identified a proximal operon putatively encoding perchlorate reductase in the genomes of two DPRB, *Dechloromonas agitata* and *Dechloromonas aromatica*. The orientation of the *pcrABCD* genes was the same in both DPRB with exception to the order of the *cld* gene (Fig. 5.27). Blast analysis of these ORFs, designated *pcrABCD*, indicated amino acid similarities to subunits of archaeal nitrate reductases and bacterial selenate reductase (*serABDC*), dimethyl sulphide dehydrogenase (*ddhABDC*), ethylbenzene dehydrogenase (*ebdABCD*), and chlorate reductase (*clrABDC*), all members of the DMSO family (Table 5.14). While the *serABDC* (16), *ddhABDC* (17), and *clrABDC* (12) operons all share the same gene order, the *pcrABCD* operon mimics the *ebdABCD* (21) operon arrangement. The significance of this observation is unknown.

#### 5.7.2 *pcrA*.

Translational analysis of the 2784 bp *pcrA* ORF identified a molybdopterin-binding domain as well as a twin-arginine signal motif of (S/T)RRXFLK (Fig. 5.27). Previous studies have suggested that the twin arginine motif tags a protein, especially one involved in electron transfer reactions, for transport to the periplasm via Sec-independent transport (Tat pathway) (17). The motif is also commonly found in electron transfer proteins possessing molybdopterin cofactors and iron-sulphur centres (16). Since the perchlorate reductase from GR-1 was located in the

periplasm and contained molybdenum and iron-sulfur centers (59), the presence of this signal peptide further supports the identification of the *pcrA* gene as encoding for the  $\alpha$ -subunit of the perchlorate reductase. In addition, the calculated molecular mass for the PcrA subunit is 105 kDa, a value that corresponds well with the 95 kDa value predicted for the  $\alpha$ -subunit of the purified perchlorate reductase from GR-1 (59).

### 3.7.3 pcrB.

Translational analysis of the 1002 bp *pcrB* ORF indicated the presence of iron-sulphur binding domains as well as four cysteine-rich clusters, a feature shared with  $\beta$ -subunits of type II DMSO enzymes (Fig. 5.28). Based on data from the  $\beta$ -subunit of the *E. coli* nitrate reductase (45), these Fe-S centres may be responsible for electron transfer to the molybdopterin-containing  $\alpha$ -subunit of perchlorate reductase. The predicted N-terminal aa sequences for the *D. agitata* and *D. aromatica* PcrB proteins were aligned with the N-terminal sequence of the purified PcrB from GR-1 (59). This alignment reinforces the identity of the *pcrB* gene (Fig. 5.28). The predicted *D. agitata* PcrB N-terminus shared 10 identical residues while the predicted *D. aromatica* PcrB shared 16 of the 18 residues reported for the purified perchlorate reductase  $\beta$ -subunit from strain GR-1. Since no signal sequence was detected, the  $\beta$ -subunit of perchlorate reductase is likely to be translocated with the  $\alpha$ -subunit in a manner similar to that proposed for the selenate reductase (62), dimethyl sulphide dehydrogenase (72), and chlorate reductase (38) enzymes. The calculated molecular mass for the PcrB subunit was 37 kDa, a value similar to the 40 kDa reported for the  $\beta$ -subunit of the purified perchlorate reductase from GR-1 (59).

### 5.7.4 pcrC.

Although a  $\gamma$ -subunit was not detected in the enzyme analysis of the perchlorate reductase from strain GR-1, a third cytochrome-type subunit responsible for connecting the reductase to the membrane was believed to have been lost during purification of the enzyme (59). This observation is borne out by our identification of an 711 bp ORF immediately downstream of the *pcrB* gene in both *D. aromatica* and *D. agitata* that exhibited sequence similarity to a type-*c* cytochrome (Table 5.14), possibly implicating the involvement of this subunit in shuttling electrons from a membrane quinone pool to the periplasmic PcrAB complex during perchlorate reduction (Fig. 5.29). The lack of amino acid sequence similarity between PcrC (ca. 25 kDa) and other type II DMSO  $\gamma$ -subunits was not surprising due to the overall sequence diversity noted in the SerC, EbdC, DhdC, and ClrC subunits (38).

### 5.7.5 pcrD.

Based on sequence identity with SerD, DdhD, EbdD, and NarJ, the final 714 bp *pcrD* ORF (ca. 25 kDa) likely encodes a system-specific molybdenum chaperone protein (Table 5.14). This finding is supported by the absolute requirement of molybdenum for active perchlorate reduction (22). The proteins SerD, DdhD, and EbdD are believed to be involved in the assembly of the mature molybdenum-containing selenate reductase, dimethyl sulphide dehydrogenase, and ethylbenzene dehydrogenase enzymes respectively prior to periplasmic translocation via the Tat pathway (62, 72, 88). However, these proteins are not believed to be part of the active enzymes.

### 5.7.6 Expression and deletion of *pcrA*.

Both expression analysis and mutagenesis of the *pcrA* gene verified the identity of the *pcrABCD* operon. Northern analysis of the *D. agitata pcrA* gene indicated a single ~ 2.7 kb expression

product in the anaerobic perchlorate and chlorate grown cultures (Fig. 5.30). However, the presence of perchlorate, chlorate, or nitrate was not enough to induce *pcrA* expression in aerobic cultures and, as such, indicates the ability of oxygen to completely inhibit *pcrA* expression as suggested by the previously documented inhibitory effects of oxygen on perchlorate reduction (22, 83) (Fig. 5.30). The 2.7 kb product corresponded to the size of the *pcrA* gene alone, which may be indicative of transcriptional processing within the operon.

Functional proof that the *pcrA* gene is involved in perchlorate reduction was demonstrated by mutational knockout in *D. aromatica* in which replacement of the *pcrA* gene by homologous recombination with a tetracycline resistance gene abolished both perchlorate and chlorate reduction (Fig. 5.31). However, as expected, the  $\Delta$ *pcrA* mutant *D. aromatica* strain was still able to grow aerobically (data not shown) as well as anaerobically via nitrate reduction, indicating separate metabolic pathways for each electron acceptor (Fig. 5.31).

#### 5.7.7 Phylogenetic analysis of PcrA.

Based on the biochemical analysis of the purified enzyme from strain GR-1 (59), perchlorate reductase was assigned as a member of the type II DMSO family (73). Our sequence analysis of the perchlorate reductase genes also supports this designation. Enzymes within the prokaryotic type II DMSO family reside in the periplasm and share a common molybdenum cofactor known as bis(molybdopterin guanine dinucleotide) Mo (61, 72, 73). DMSO enzymes are involved in a myriad of reduction capabilities including the dissimilatory reduction of toxic elements such as selenate and arsenate (73). Using  $\alpha$ -subunit protein sequences from known DMSO enzymes (72, 73) and from the PcrA sequences resulting from this study, a phylogenetic tree was constructed (Fig 5.32) with a topology similar to a DMSO reductase family tree constructed by McEwan and coworkers (73). Our analysis indicated that the PcrA forms its own monophyletic group within the DMSO enzymes. Based on the tree, PcrA appears to be more closely related to the NarG from *B. subtilis* and *E. coli* than to the ClrA from *I. dechloratans* further implicating the differences between the perchlorate and chlorate reductase. The PcrA may also share a common ancestor with the *E. coli* and *B. subtilis* NarG, the *I. dechloratans* ClrA, the *T. selenatis* SerA, the *R. sulfidophilum* DdhA, and the *Azoarcus* sp. EB1 EbdA, all Type II members of the DMSO family. More PcrA sequences are needed before further evolutionary conclusions can be drawn.

The distance between the perchlorate and chlorate reductase is indicative of distinct enzymes, which was supported by our molecular probing of gDNAs from perchlorate- and chlorate-reducers as well as close relatives unable to reduce either electron acceptor (Fig. 5.33). This slot-blot analysis resulted in hybridization signals for the *pcrA* gene from perchlorate reducers alone. No signal was present in the close-relatives or *Pseudomonas* sp. strain PK, *Dechloromarinus chlorophilus*, and *Ideonella dechloratans*, organisms capable of only chlorate and not perchlorate reduction (Fig. 5.33). This supports the evolution and existence of two distinct metabolic pathways involved in the reduction of these analogous compounds.

From the current study, it is clear that a more complete understanding of perchlorate reduction and other environmentally significant pathways will be pivotal in providing knowledge applicable to the design of future bioremediative strategies. Perchlorate reductase and other members of the type II DMSO family play a role in a broad range of substrate reductions with varying active sites comprising the major differences between family members. These differing

active sites indicate a common reductase ancestor which acquired mutations advantageous to the utilization of specific substrates. Thus, it is possible that directed mutagenesis of the active site of DMSO enzymes could lead to the creation of novel enzymes useful for biotechnological as well as bioremediative application.

### **5.8. The chlorite dismutase gene of *Dechloromonas agitata*: Sequencing, transcriptional analysis and its use as a metabolic probe**

The dismutation of chlorite into chloride and O<sub>2</sub> represents a central step in the reductive pathway of perchlorate that is common to all dissimilatory perchlorate-reducing bacteria. Chlorite dismutation is mediated by a highly conserved single enzyme, chlorite dismutase. The chlorite dismutase gene, *cld*, was isolated and sequenced from the perchlorate-reducing bacterium *Dechloromonas agitata* strain CKB. Sequence analysis identified an open reading frame of 834 bp with a predicted N-terminal sequence of the mature protein identical to that of the previously-purified *D. agitata* chlorite dismutase enzyme. The predicted translation product of the *D. agitata cld* gene is a protein of 277 aa including a leader peptide of 26 aa. Primer extension analysis revealed the presence of an AT-rich DNA region that could represent the -10 promoter region of the *D. agitata cld* gene. Northern blot analysis indicated that the *cld* gene was transcriptionally up-regulated when *D. agitata* cells were grown in perchlorate-reducing versus aerobic conditions. Slot blot hybridizations with a *D. agitata cld* probe demonstrated the conservation of the *cld* gene among perchlorate-reducing bacteria. This study represents the first description of a functional gene associated with microbial perchlorate reduction.

#### 5.8.1 *Ideonella dechloratans* chlorite dismutase primers.

As outlined in the introduction, the gene sequence for CD (*cld*) from *Ideonella dechloratans* was recently submitted to the GenBank database (accession number AJ296077), however, no information regarding this gene is currently available in the literature. Because it was not known which part of the putative *I. dechloratans* chlorite dismutase gene contained the most highly conserved sequence region, a series of primers were designed to specifically amplify the 5' end, central portion and 3' end of the chlorite dismutase gene from *I. dechloratans* (Table 5.14). Each primer set was used in a PCR reaction using either *I. dechloratans* or *D. agitata* gDNA as the template. PCR amplification of *I. dechloratans* DNA using the primer set ICD-283F and ICD-1140R yielded an 857 bp product spanning the central region of the putative coding sequence for CD. Using the primer set ICD-283F and ICD-713R, a 430 bp product was produced corresponding to the 5' end of the *cld* gene. A 399 bp product corresponding to the 3' end of the *cld* gene was amplified using the primer set ICD-741F and ICD-1140R. In contrast, no amplification products of the desired length were obtained with any of the primer sets using *D. agitata* DNA as the template.

#### 5.8.2 Southern blot and library screening.

To determine if the *I. dechloratans cld* gene sequence could be used to screen a *D. agitata* genomic library, a Southern blot was performed using the labeled amplification products of *I. dechloratans* (see above) as probes. Using the probe corresponding to the 3' end of the *I. dechloratans cld* gene, a positive signal was visible in each lane of digested *D. agitata* gDNA (data not shown). However, no hybridization signal resulted when the same Southern blot was hybridized with the probe corresponding to the 5' end of the *I. dechloratans* CD gene (data not shown). This result indicates that the 3' end of the *cld* gene is more conserved than the 5' end

and likely encodes the protoheme IX group binding region observed in the mature proteins from *I. dechloratans* (103) and the DPRB strain GR-1 (123), which is part of the active site of the chlorite dismutase enzyme. As such, the *I. dechloratans* probe corresponding to the 3' end of the *cld* gene was used in all subsequent screening experiments.

Given the positive results from the Southern blot, a *D. agitata* gDNA lambda library was constructed and screened using the *I. dechloratans cld* probe. Screening of approximately 1200 plaques resulted in a single positive clone. Following rescreening, the phagemid was produced and digested with *EcoRI* to yield an insert of approximately 7.5 kb.

### 5.8.3 Sequence analysis.

Previously we purified and partially characterized the chlorite dismutase enzyme from *D. agitata* (31). The purified CD was a homotetramer with a molecular mass of 120 kDa and a specific activity of 1,928  $\mu\text{mol}$  chlorite dismutated per mg of protein per minute (31). This is similar to the molecular mass and specific activity observed for the CD previously purified from the perchlorate-reducing bacteria strain GR-1 (123) and *I. dechloratans* (103). Commercial N-terminal sequencing (Commonwealth Biotechnologies Inc., Richmond VA) of the CD purified from *D. agitata* revealed the following twenty amino acid sequence in the mature protein: DAKPPMAMPDMTKILTAPGV.

Sequencing of the *D. agitata* insert revealed an open reading frame (ORF) of 834 bp with a predicted N-terminal sequence of the mature protein identical to that of the purified *D. agitata* chlorite dismutase (Fig. 5.34). In addition, sequence similarity to the putative *I. dechloratans cld* gene sequence provided further evidence that this ORF encodes the *D. agitata* CD protein (Fig. 2). BLAST 2.2 analysis (5) indicated that the *D. agitata* CD sequence was 71% similar to *I. dechloratans* chlorite dismutase at the amino acid (aa) level. No other proteins in the GenBank database were more than 24% similar to the product encoded by the *D. agitata cld* gene, emphasizing the unique nature of the chlorite dismutase enzyme.

The predicted translation product of the *D. agitata cld* gene is a protein of 277 aa including a leader peptide of 26 aa (Fig. 5.34). The *D. agitata* CD amino acid sequence was manually aligned to the *I. dechloratans cld* gene product and, based on this very limited comparative analyses, regions of aa sequence conservation could be identified within the CD protein (Fig. 5.35). As initially indicated by the Southern blot analysis, very low sequence similarity exists at the N-terminus of the two proteins. In addition, no common promoter region could be distinguished from alignment analysis. However, regions of conservation in the central and C-terminus of the protein were identified that could be indicative of an active site or catalytic domain for the chlorite dismutase protein. In support of this, a previous study demonstrated that a CD-specific immunoprobe raised against the purified CD from *D. agitata* bound to all DPRB screened regardless of their phylogenetic affiliation, however, variable binding was observed suggesting that minor differences in the mature CD protein do exist (84). Comparative analysis with more chlorite dismutase sequences are needed to refine our current view of sequence conservation and functional motifs for this protein.

#### 5.8.4 Transcription start-site analysis of the CD gene.

Because no data is available regarding promoter structure in the beta Proteobacterium *D. agitata*, primer extension reactions were performed to identify the *cld* gene promoter region in *D. agitata*. Reactions contained total RNA purified from a *D. agitata* culture grown under anaerobic conditions with  $\text{ClO}_4^-$  as the electron acceptor. Primer CD107RC, designed to target the 5' end of the CD mRNA, yielded a single extension product (Fig. 5.36). From the primer extension data, a putative -10 promoter region was identified (5'-AAATTT-3') that is located 8 bp upstream of the transcription start site (Fig. 1). The sole transcriptional start site detected by primer extension indicates that the *cld* gene encodes a 277 aa product and that transcription begins 164 bp upstream of the DNA region encoding the N-terminal sequence as determined from the purified *D. agitata* CD protein (31).

Prior to the N-terminus of the mature CD protein, there is a region of DNA that, when read in-frame, could encode a 26 aa peptide (Fig. 5.34). This intervening amino acid sequence may correspond to a signal peptide for the chlorite dismutase protein that could play a role in targeting the chlorite dismutase to the bacterial cell membrane (18, 31, 84). In support of the membrane-bound nature of the chlorite dismutase enzyme, previous biochemical studies demonstrated that the CD activity was associated with both the cell membrane and soluble fractions of a lysed-cell preparation of *D. agitata* when prepared using a French press (18, 31) which suggested that the CD was loosely bound to the membrane or was present in the periplasm. In *I. dechloratans*, the CD enzyme activity was located primarily in the periplasmic extract (103). Furthermore, the CD specific immunoprobe readily bound to whole cells of both of these organisms suggesting that the antigenic portion of the CD was present on the outer membrane (84). In contrast, van Ginkel and coworkers (1996) claimed that the CD activity of the DPRB, strain GR-1, was located exclusively in the soluble fraction, however, no attempt was made to separate out the periplasmic fractions from the soluble fractions in that study (123).

The identification of a ribosome binding site (RBS) located immediately upstream of the potential leader peptide (Fig. 5.34) lends support to the hypothesis that the leader peptide of the *D. agitata cld* gene is translated and later removed to produce the mature CD protein as determined from the N-terminal sequence of the purified CD enzyme. The RBS has a sequence of 5'-AGAAAGG'3' which is the exact reverse complement of the conserved terminal 3' end of the bacterial 16S rRNA.

#### 5.8.5 Protein motifs and signal peptide.

Both the entire and mature putative protein sequences for the *D. agitata* CD were submitted to the PredictProtein server ([http://cubic.bioc.columbia.edu/predictprotein/submit\\_def.html](http://cubic.bioc.columbia.edu/predictprotein/submit_def.html)). This server submits protein sequences to a number of modeling programs available online (98). Interestingly, the CD sequence did not match any sequence currently in the protein data bank to allow for 3D modeling. As such, homology modeling was not possible for either the entire or mature CD sequence. However, a secondary structure was predicted with a 44% alpha helix, 16% beta sheet, and 40% loop region. The “DAS” transmembrane prediction server (36) found only one likely transmembrane region in the sequence (Fig. 5.34). This hydrophobic region starts at aa 6 and ends at aa 11 (GLLLTFMALLSV), and corresponds to a putative helix located in the leader sequence. The results from the SignalP server (80) further indicated that the leader

sequence was indeed a signal peptide with a characteristically positively-charged N terminus, a hydrophobic helix and a cleavage site motif of SQA-QQA (Fig. 5.34) (7).

#### 5.8.6 Transcriptional regulation of chlorite dismutase.

Previous physiological studies on the environmental factors that influence microbial perchlorate reduction demonstrated that perchlorate reduction was dependent on the presence of the CD enzyme which was induced during the anaerobic metabolism of perchlorate (22). In that study, CD expression was negatively regulated by oxygen and nitrate even in the presence of perchlorate (22). To characterize the transcriptional regulation of the *D. agitata cld* gene, a Northern blot containing RNA from *D. agitata* grown with O<sub>2</sub> or ClO<sub>4</sub><sup>-</sup> was performed using a *D. agitata* probe that corresponded to the 3' end of the *cld* gene. Hybridization analysis detected a band of approximately 950 bases in both RNA samples (Fig. 5.37). A faint band was evident in the aerobic culture but an intense signal was obtained from the RNA of the perchlorate-grown culture, indicating that the *cld* gene is expressed at basal levels under aerobic conditions but that transcription of this gene is greatly increased when the cells are grown under perchlorate-reducing conditions. This result supports the physiological studies and correlates well with the results obtained using the CD-specific immunoprobe which only bound to the *D. agitata* cells grown under perchlorate-reducing conditions and failed to bind to cells from an aerobic culture (84). Although a faint signal was detected in the Northern blot from aerobic *D. agitata* cultures using the *cld* probe, it is possible that either the amount of CD protein produced from this basal-level transcription is below the limit of detection by the immunoprobe or that there may be additional genetic regulation of chlorite dismutase at either the translational or post-translational level.

It is also apparent from the Northern blot results that the chlorite dismutase gene is not transcribed as part of an operon. Only a single band representing a mRNA of approximately 950 bp was detected. Since the length of the *D. agitata cld* gene from the transcription start site to the stop codon is 921 bp, this suggests that the only ORF contained on this transcript is that for chlorite dismutase. Therefore, it appears as though the *cld* gene is transcribed independent of other genes such as perchlorate reductase that are also involved in this metabolic pathway.

#### 5.8.7 Utility of *D. agitata* CD gene probe.

Microbial dissimilatory perchlorate reduction is a phylogenetically diverse metabolism and DPRB isolates have been placed in four of the five subclasses of the Proteobacteria (31). As such, DPRB molecular probes based on signature nucleotide sequences within the 16S rDNA sequence are of limited use. Furthermore, 16S rDNA sequence analysis indicates that several DPRB show less than 0.5 % divergence in 16S rDNA sequence with non-perchlorate-reducing relatives (1, 2). To determine the utility of the *D. agitata cld* gene as a metabolic probe, gDNAs of several phylogenetically distinct DPRB representing the alpha, beta, and gamma subclasses of the Proteobacteria were screened in a slot-blot assay (Fig. 5.38). Organisms closely related to the DPRB which do not grow by dissimilatory perchlorate reduction were also included in the analysis. All the DPRB tested positive regardless of phylogenetic affiliation using the *D. agitata* probe to the 3'-end of the *cld* gene (Fig. 5.38a). Strong hybridization signals were obtained from the DPRB *Azoarcus* strain LT-1, *I. dechloratans*, and *D. agitata*. Obvious signals were also obtained from the DPRB *Dechlorospirillum* strain WD, *Dechloromarinus* strain NSS, and *Dechlorosoma suillum* while weak, but still visible, signals were obtained from the gDNAs

of the other DPRB. No signal was obtained from the non-perchlorate reducers *Rhodocyclus tenuis*, *Dechloromonas* strain JJ, *Pseudomonas stutzeri*, and *E. coli* (Fig. 5.38a).

Differences in hybridization intensities for the DPRB indicate that conservation in the *cld* gene sequence varies among the perchlorate-reducing bacteria and that these differences do not reflect the phylogeny of these organisms as determined from 16S rDNA analysis. In support of this, a similar variance in signal response based on dot-blot intensity was observed in studies with the CD-specific immunoprobe for the various DPRB suggesting that some minor differences exist in the mature CD protein in these organisms (84).

Predictably, and similarly to observations made with the immunoprobe studies (84), the most intense signal resulted from the positive control *D. agitata*. *Rhodocyclus tenuis*, a phylogenetically close non-perchlorate reducing relative to *D. agitata* (1, 18, 31), did not result in any visual hybridization signal. Similarly, *Dechloromonas* strain JJ, the only known *Dechloromonas* species that is incapable of perchlorate reduction (27) and the closest non-perchlorate reducing relative to the DPRB *D. aromatica* strain RCB, was also negative. Similar results were observed with the DPRB *Pseudomonas* strain PK and its closest non-DPRB relative, *Ps. stutzeri*, although these organisms share over 99% 16S rDNA sequence similarity (31).

Interestingly, a weak hybridization signal was obtained from *Magnetospirillum magnetotacticum*, a bacterium closely related to the DPRB *Dechlorospirillum anomalous* strain WD (2, 31, 32). This was unexpected as previous studies in our lab demonstrated that several *Magnetospirillum* species including *M. magnetotacticum* did not grow by dissimilatory perchlorate reduction (32). A subsequent search of the *M. magnetotacticum* genome (URL: <http://genome.ornl.gov/microbial/mmag/>) revealed the presence of a putative chlorite dismutase gene. The physiological ramifications of this discovery are as yet unknown.

#### 5.8.8 Significance

Chlorite dismutase has been shown to be a central enzyme involved in microbial perchlorate reduction (31, 104, 123). The present study describes the identification and characterization of the *cld* gene encoding chlorite dismutase in *D. agitata*. As such, this is the first description of a functional gene associated with microbial perchlorate respiration, a ubiquitous metabolism in the environment (31). Previous studies indicated that the CD enzyme is unique to DPRB and is highly conserved amongst the phylogenetically diverse DPRB (31, 84). In addition, close relatives of the known DPRB which cannot grow by perchlorate respiration are incapable of the dismutation of chlorite (18, 31). In support of this, the chlorite dismutase gene probe developed in this study hybridized to all DPRB tested regardless of their phylogenetic affiliation. In general, the gene probe was specific to DPRB and cross hybridization with non-DPRB was not observed, regardless of their phylogenetic similarity. However, one non-DPRB, *M. magnetotacticum*, did hybridize to the gene probe and examination of the *M. magnetotacticum* genome sequence identified the presence of a putative *cld* gene. As previous physiological studies with this and other *Magnetospirillum* species indicated that they could not grow by perchlorate or chlorate respiration (32), the functional role of the *cld* gene in this organism is unknown.



Similarly to previous observations made in studies on the environmental factors that influence microbial perchlorate reduction (22), the transcriptional regulation analyses indicated that the *cld* gene was up-regulated to a significant extent when the organism was grown on perchlorate as opposed to aerobic growth. This result indicates the potential of a chlorite dismutase gene probe for monitoring active perchlorate metabolism by mixed DPRB populations in environmental samples during a bioremediative process.

## 6.0 Conclusions

The field of microbial perchlorate reduction has clearly advanced significantly in a very short period from a poorly understood metabolism to a burgeoning scientific field of discovery. As outlined above, our studies have resulted in a much greater appreciation of the microbiology, biochemistry and genetic systems involved and the application of this to the successful treatment of contaminated environments. Overall, the future is promising, however, research in this field is still in its infancy. Nothing is known of the evolutionary timeline of this metabolism. From a biogeochemical perspective, a better understanding of how perchlorate is formed in the natural environment and what geochemical conditions are required for its formation might give some insight into this. From a microbial perspective, it will be important to look for this metabolism in more extreme environments such as hypersaline or hyperthermophilic environments to obtain DPRB isolates across a broader phylogeny to establish a broad-base molecular chronometer. Our recent completion of the first draft genome sequence of a perchlorate-reducing organism, *Dechloromonas aromatica* strain RCB in collaboration with the Joint Genome Institute, CA, offers exciting new avenues of research ([http://genome.jgi-psf.org/draft\\_microbes/decar/decar.home.html](http://genome.jgi-psf.org/draft_microbes/decar/decar.home.html)). In addition to its ability to reduce (per)chlorate, *D. aromatica* possesses several other unique metabolic capabilities that have direct application to the bioremediative treatment of several contaminants, including monaromatic hydrocarbons, heavy metals and radionuclides. The availability of this genome sequence has already allowed the development of genome chips containing the entire genome of this organism (LA Achenbach and JD Coates, unpublished). Such microarrays are being used to furnish data on the environmental factors that affect the activity of DPRB and how these microorganisms function in concert with other members of a microbial community during the treatment of a contaminated site. Furthermore, continual genome annotation will allow the investigation of protein–protein interactions and substrate channeling during perchlorate reduction and also protein–DNA interactions, which will give a deeper insight into the regulatory mechanisms involved.

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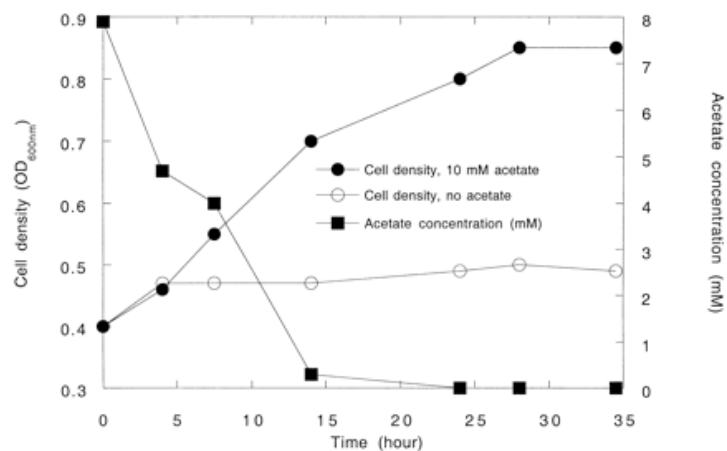
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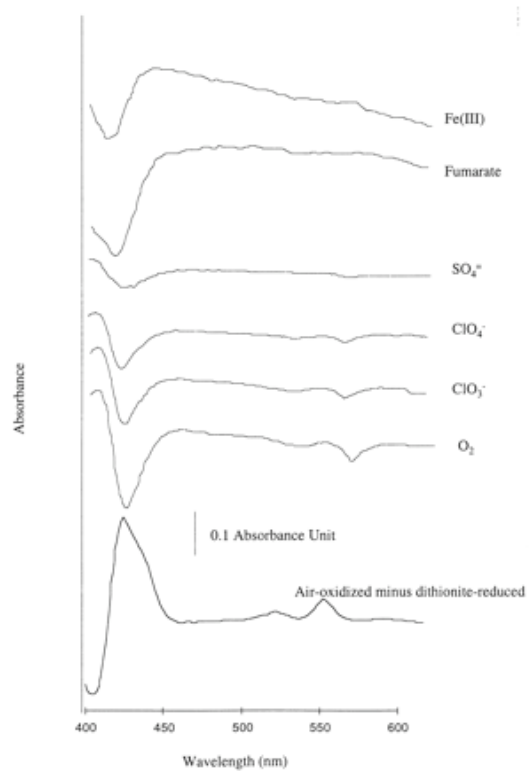
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## Appendix A (Figures)



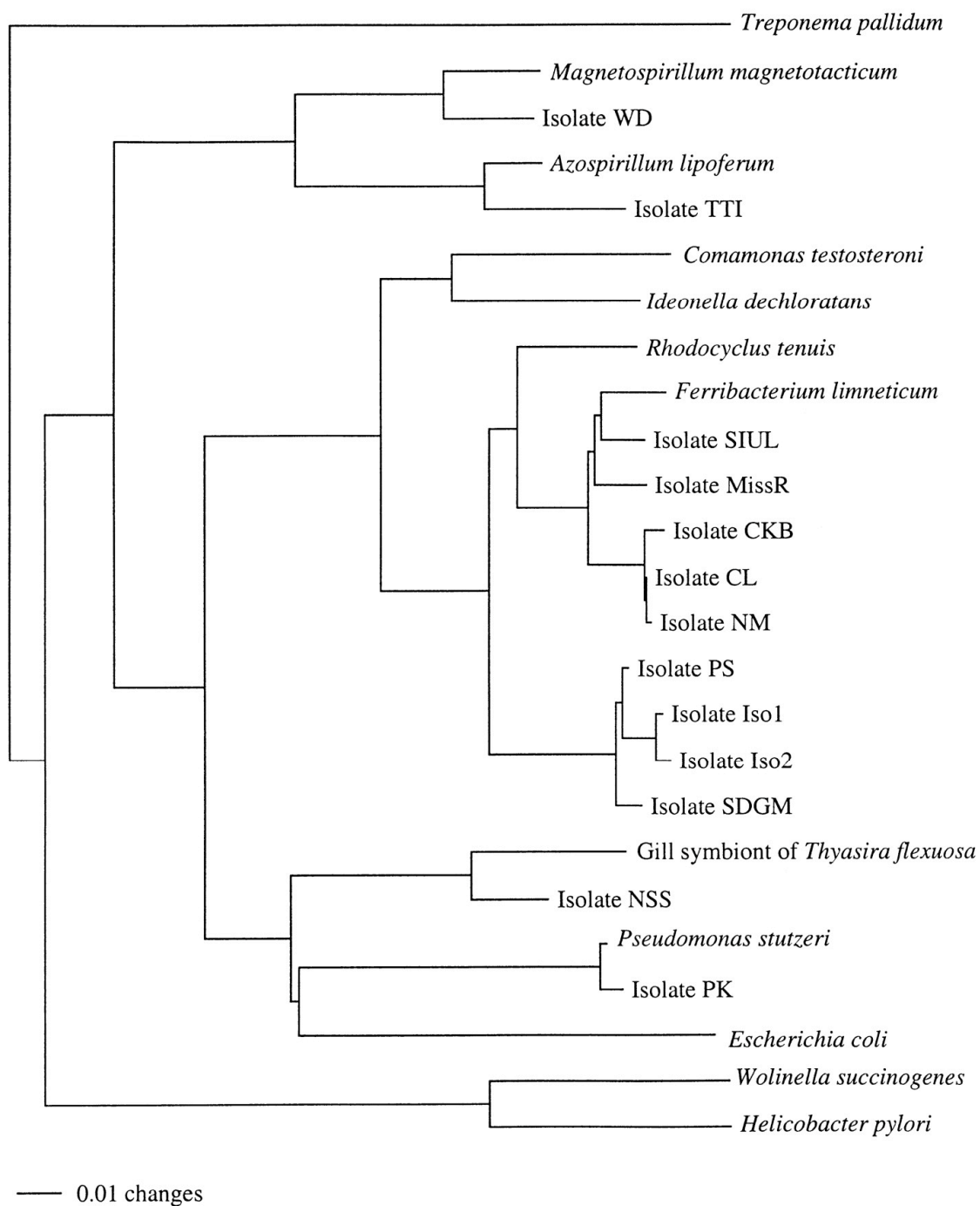
**Figure 5.1**

Growth curve for (per)chlorate-reducing strain PK with when acetate was the electron donor and chlorate (10 mM) was the sole electron acceptor. The data are averages based on triplicate determinations. OD<sub>600nm</sub>, optical density at 600 nm.



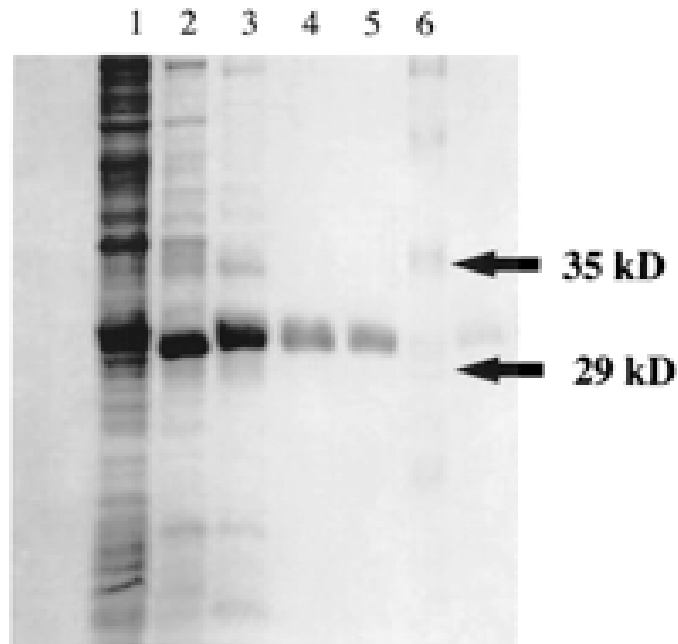
**Figure 5.2**

Difference absorbance spectra of H<sub>2</sub>-reduced washed whole-cell suspensions of (per)chlorate-reducing strain CKB in the presence of various potential electron acceptors



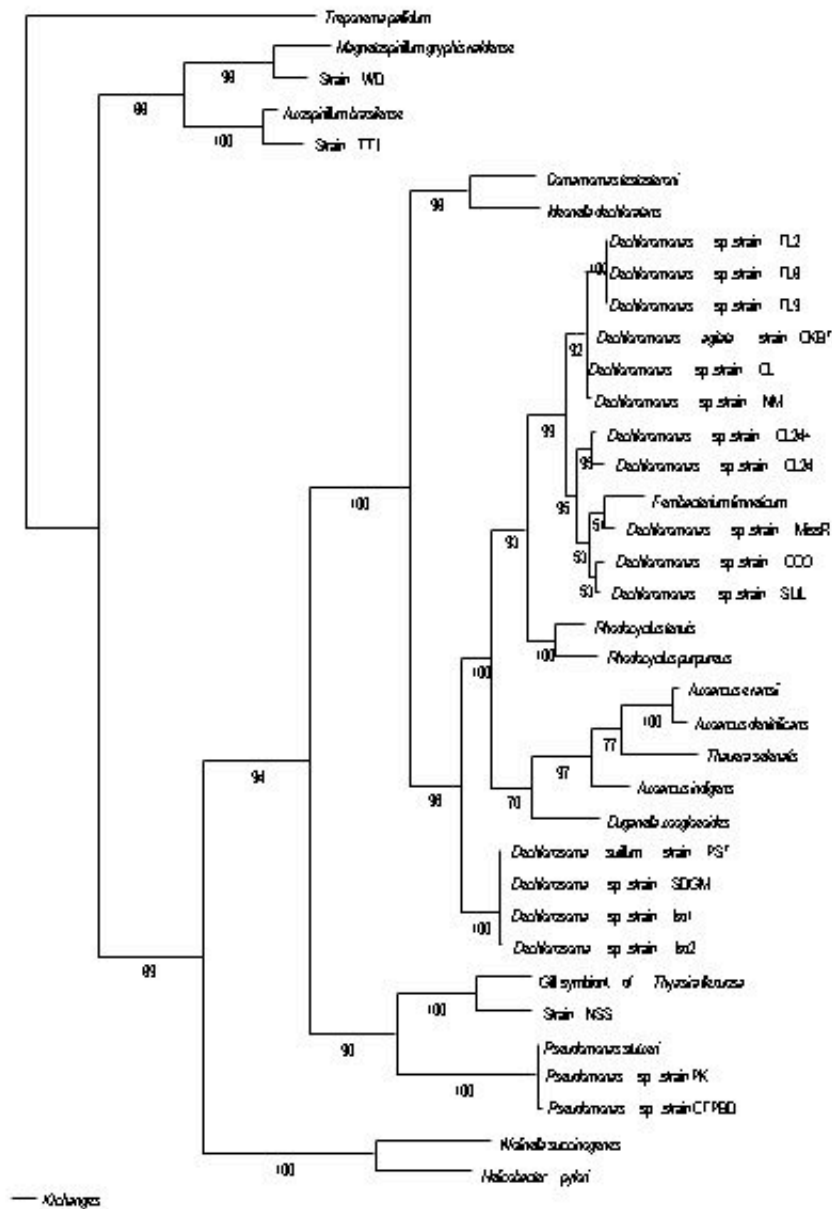
**Figure 5.3**

Phylogenetic tree based on 16S rDNA sequence data resulting from a distance analysis performed with the Jukes-Cantor correction. The same topology was obtained by using either parsimony or maximum likelihood and was supported by bootstrap analysis.



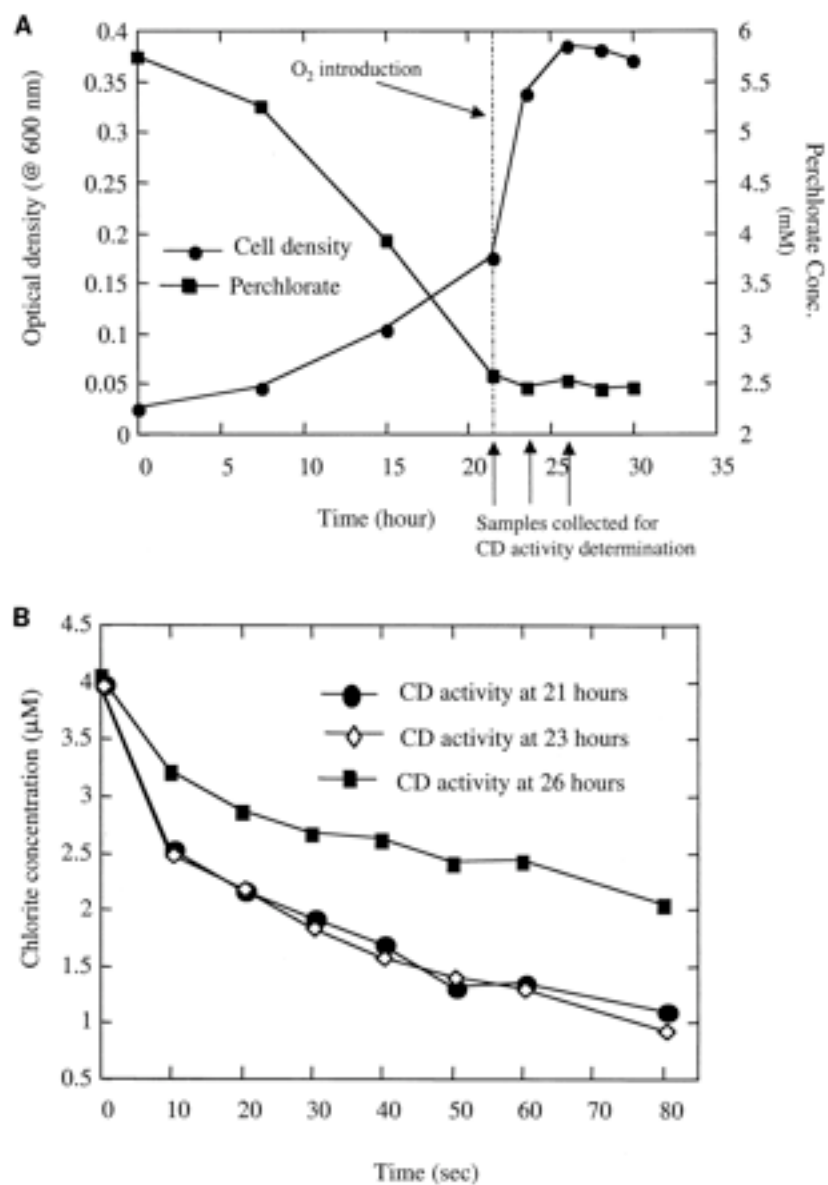
**Figure 5.4**

SDS-PAGE gel containing the chlorite dismutase active fractions from strain CKB. Lane 1, cell lysate fraction; lane 2, chlorite dismutase active pool from the Q-Sepharose column; lane 3, active pool from the hydroxyapatite column; lane 4, chlorite dismutase active fraction from the Phenyl Sepharose column; lane 5, purified chlorite dismutase from the Superdex 200 column; lane 6, molecular mass standard.



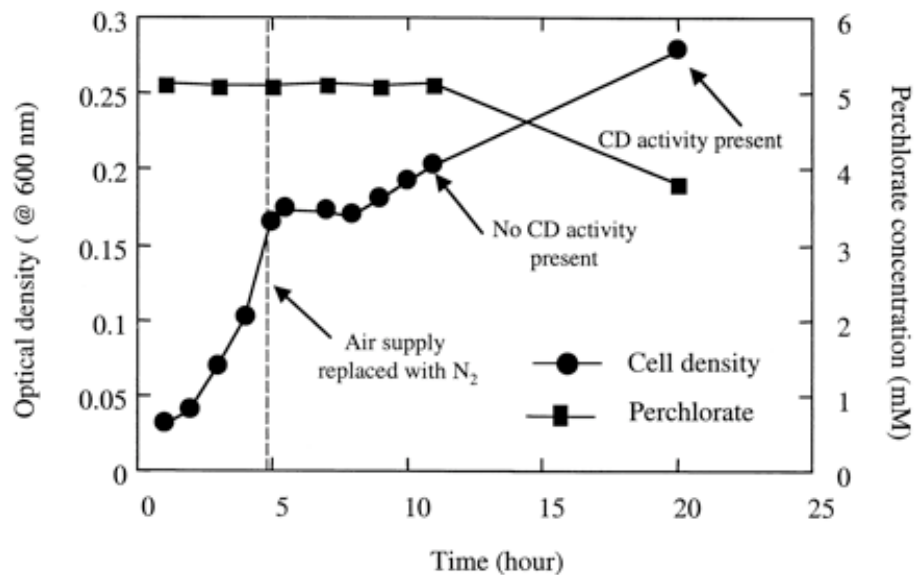
**Figure 5.5**

Phylogenetic tree of newly isolated chlorate-reducing bacteria and their closest relatives using a heuristic search with parsimony analysis. Tree length = 1205, C.I. minus uninformative sites = 0.502, R.I. = 0.742. Bootstrap support values from 100 replications are indicated for each node. This figure represents one of two most parsimonious trees with the only difference being the topology among the *Dechloromonas* sp. strains MissR, CCO, and SIUL and *F. limneticum*.



**Figure 5.6**

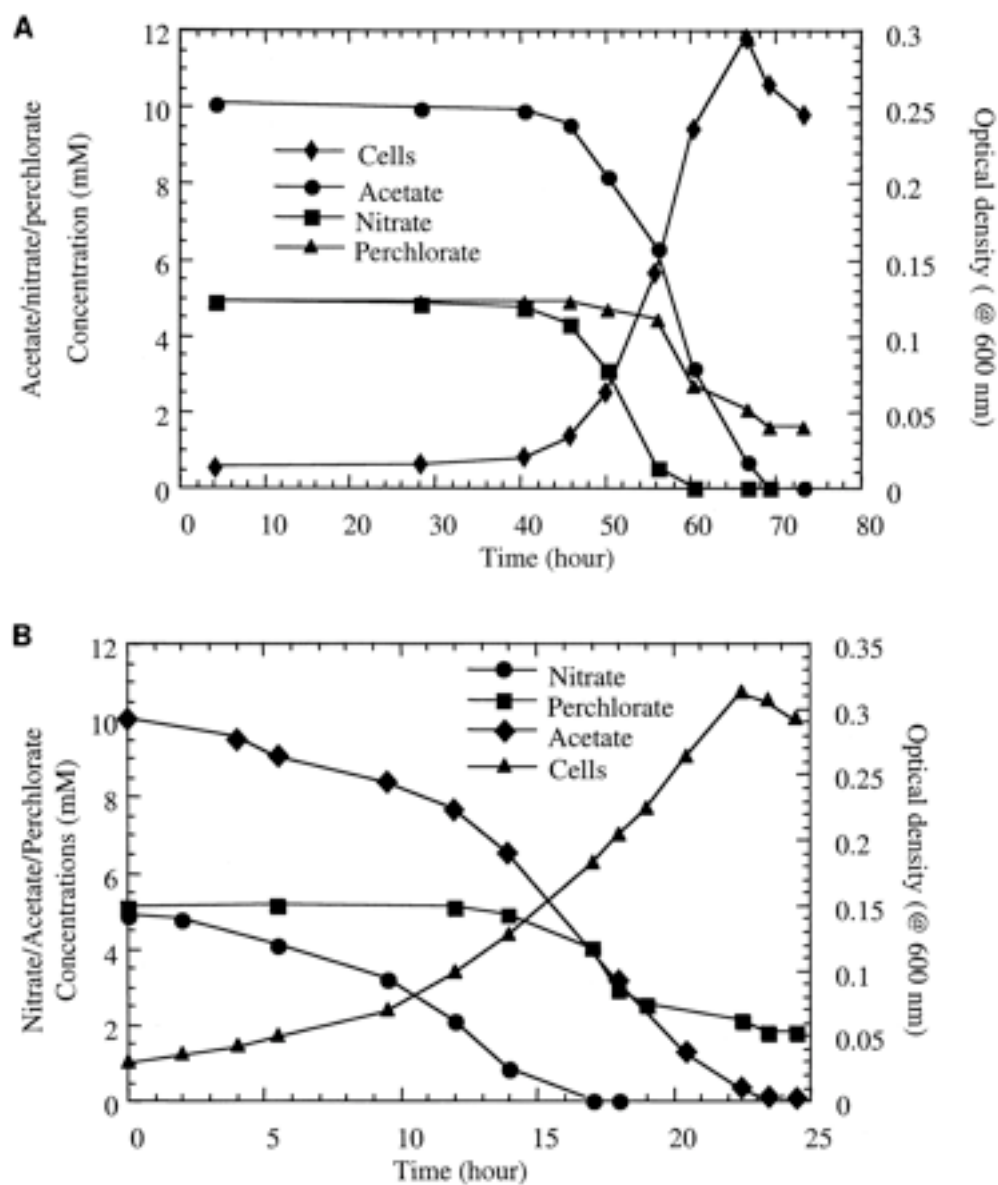
Effects of the introduction of oxygen after 21 h of incubation on growth and perchlorate reduction (A) and CD activity (B) by an active perchlorate-respiring culture of *D. suillum*.



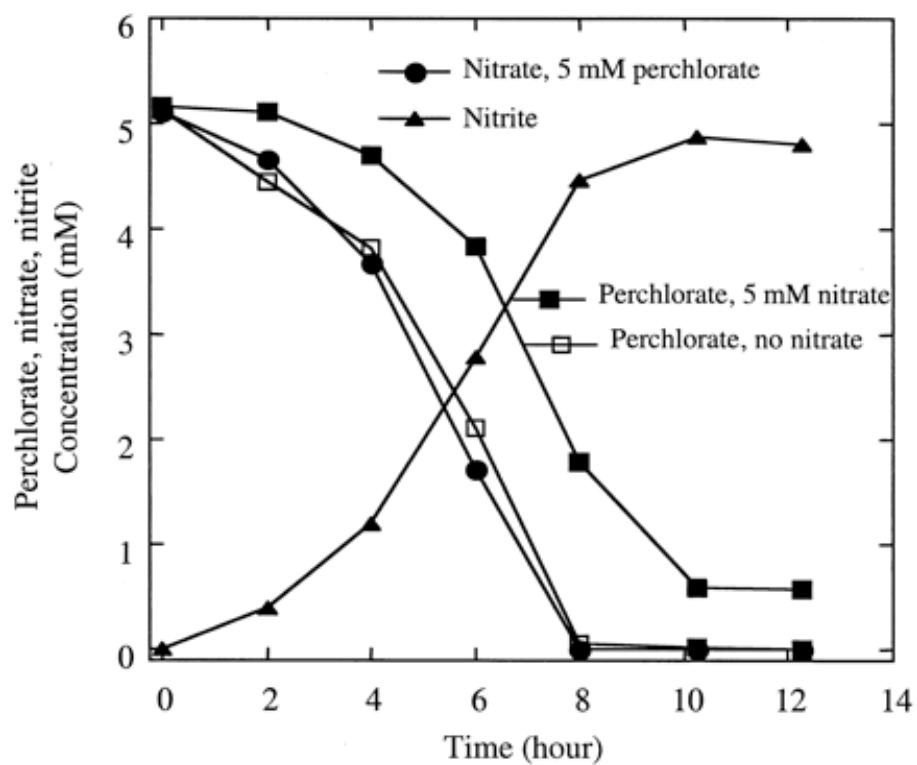
**Figure 5.7**

Effect on growth and CD activity of switching an aerobic culture of *D. suillum* amended with perchlorate to anaerobic conditions during log-phase growth.

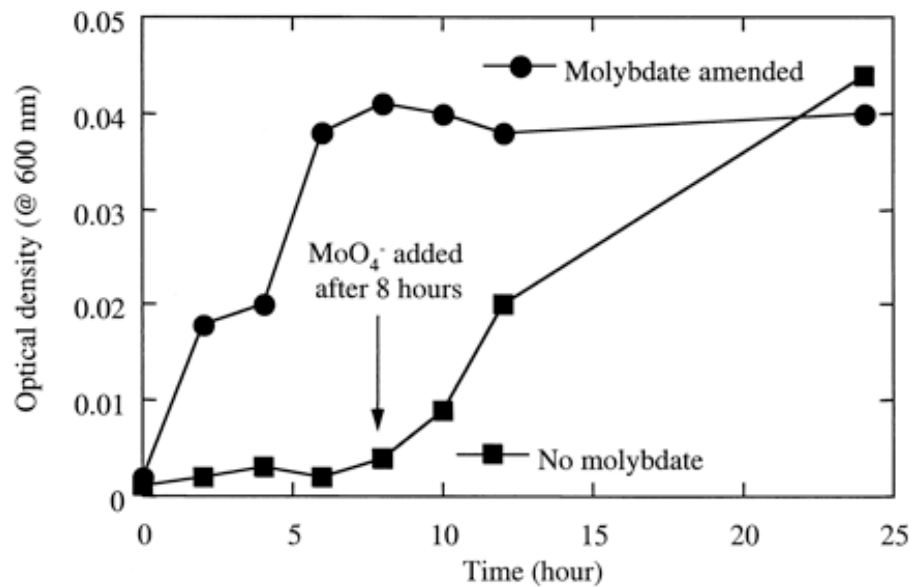




**Figure 5.8**  
Growth, nitrate, and perchlorate reduction by nitrate-grown (A) and perchlorate-grown (B) cultures of *D. suillum*.

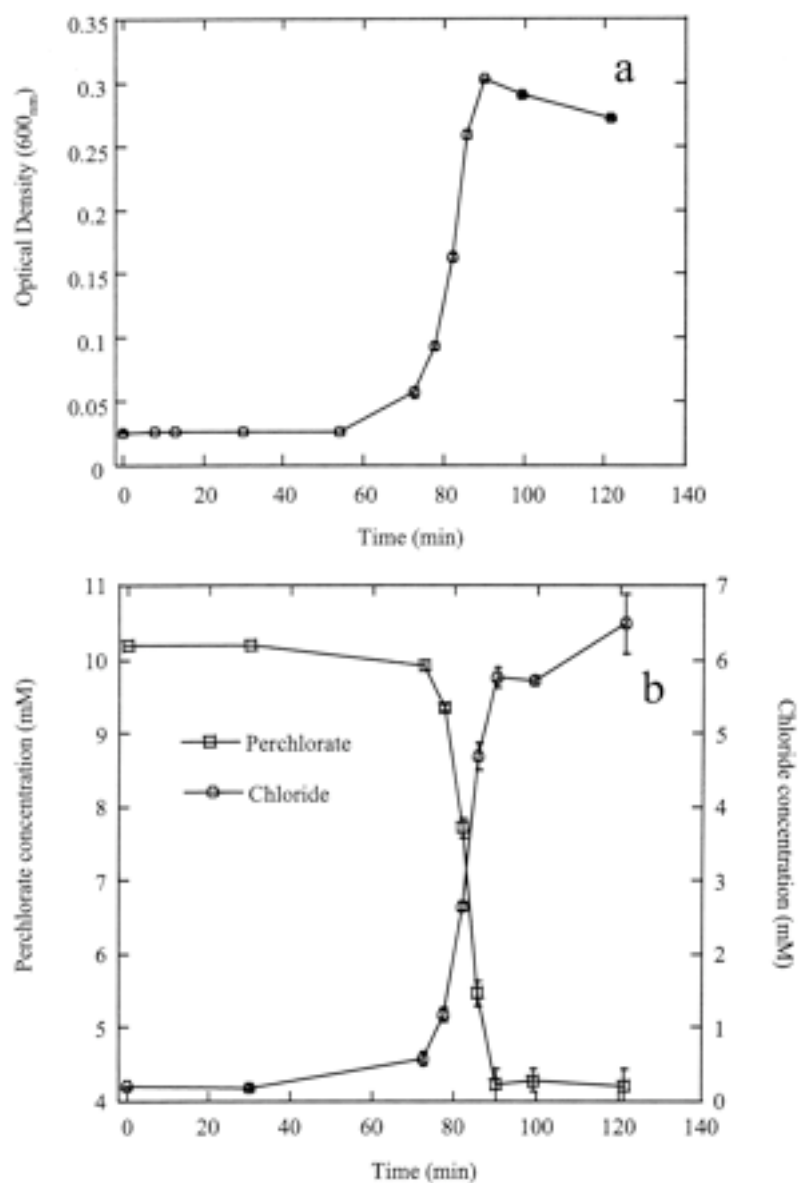


**Figure 5.9**  
Growth, nitrate and perchlorate reduction, and nitrite production by a perchlorate-grown culture of *D. agitata*.



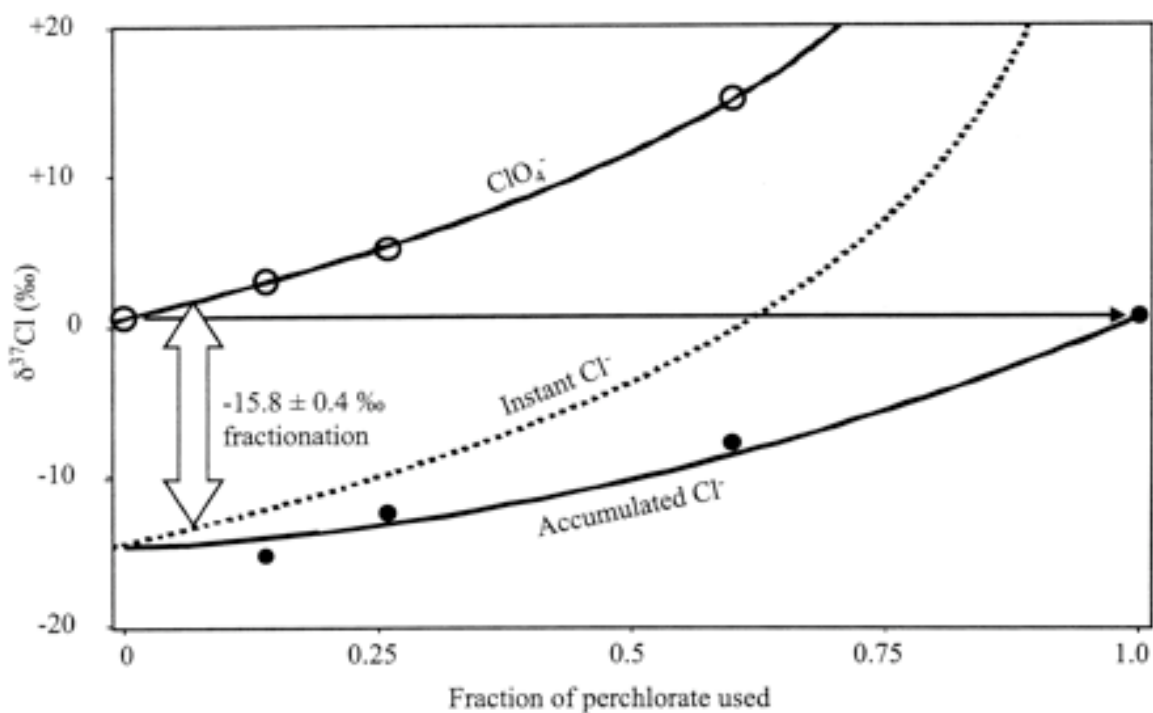
**Figure 5.10**

Anaerobic growth of *D. aromatica* on perchlorate in the presence and absence of molybdate.



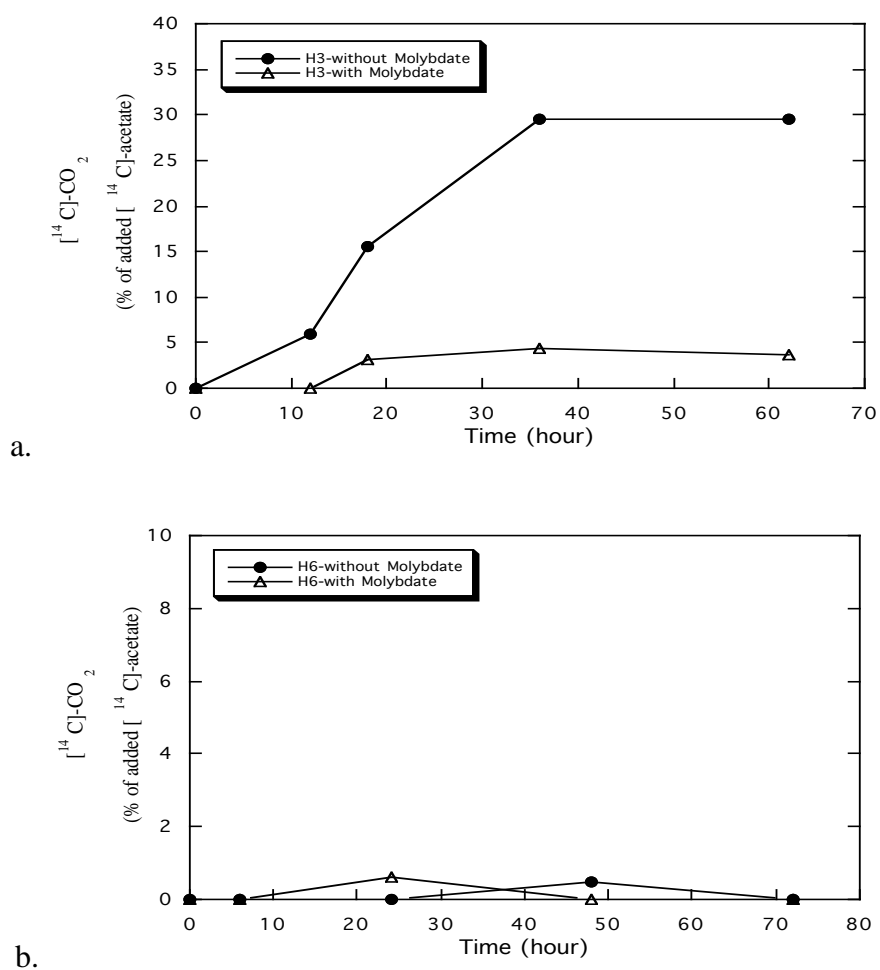
**Figure 5.11**

Cell density increase (a) and perchlorate reduction with corresponding chloride formation (b) by *D. suillum* during growth with acetate as the sole electron donor. The results depicted are the averages of triplicate determinations.



**Figure 5.12**

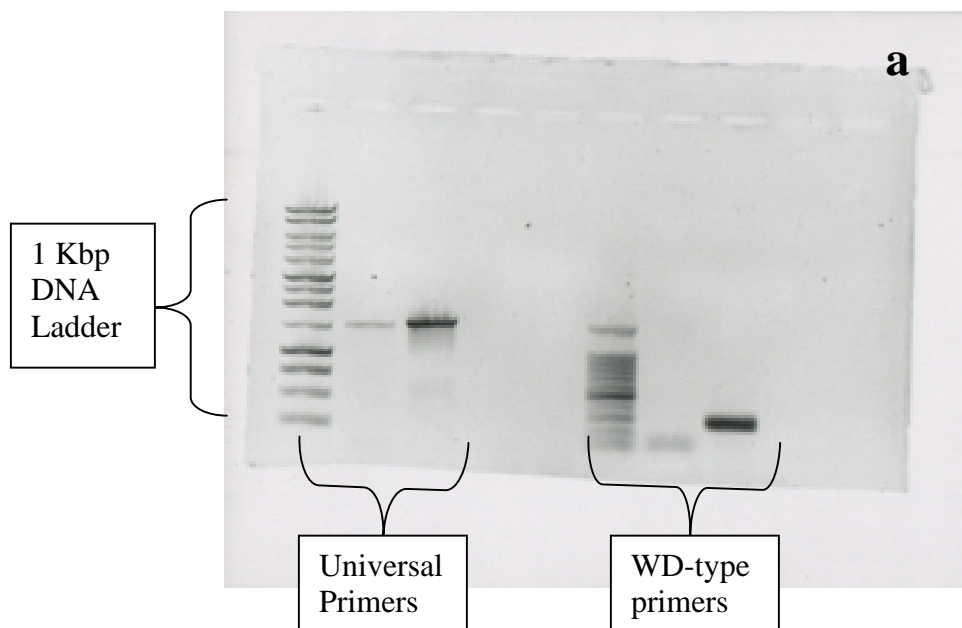
Chlorine isotope compositions of chloride and residual perchlorate during microbial reduction by *D. suillum*. Instant  $\text{Cl}^-$  indicates the calculated isotopic composition of chloride produced from the remaining perchlorate at that point in the process, as though unmixed with previously produced chloride.



**Figure 5.13**

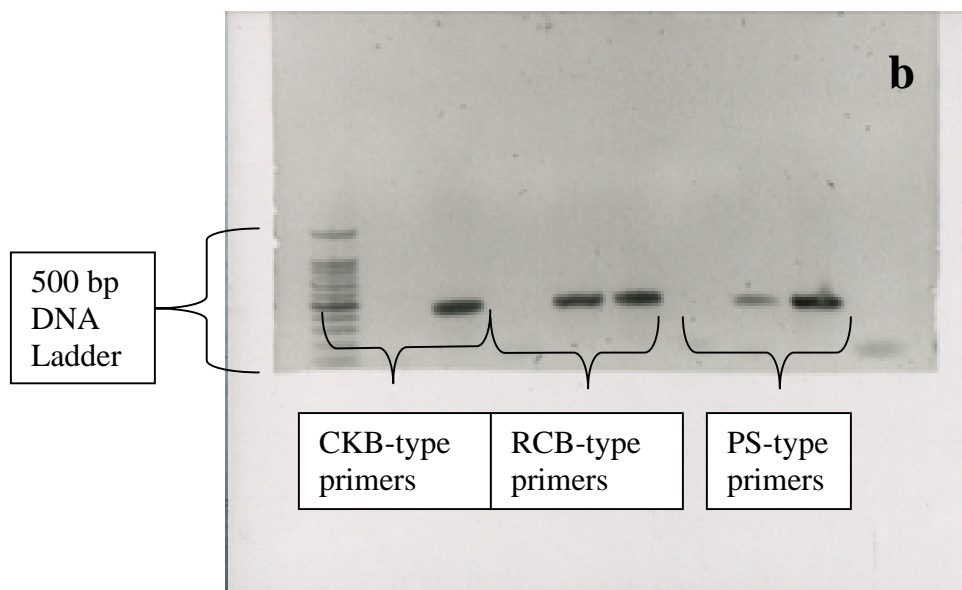
TEAP determinations for San Nicolas Island Heavily contaminated site sediments. Symbols without molybdate are the average of triplicate determinations; symbols with molybdate are the average of duplicate determinations.

- a. H3= Heavily contaminated sediment, 3 foot depth
- b. H6= Heavily contaminated sediment, 6 foot depth



**Figure 5.14**

Agarose gels with PCR products generated by the amplification of gDNA using primers specific for: 5a) Lane 1: 1Kbp DNA Ladder; lanes 2, 3, and 4: Universal 16S rDNA amplified with 8F & 1525R primer set; lane 6: 500bp DNA Ladder; and, lanes 7, 8, and 9: DPRB 16S rDNA amplified with WD-type primers (developed by Dr Laurie A. Achenbach). Lanes 2 & 7 contain gDNA extracts from an isolated DPRB (LT-1); Lanes 3 & 8 are positive controls for each primer set; and, lanes 4 & 9 are negative (no gDNA) controls.

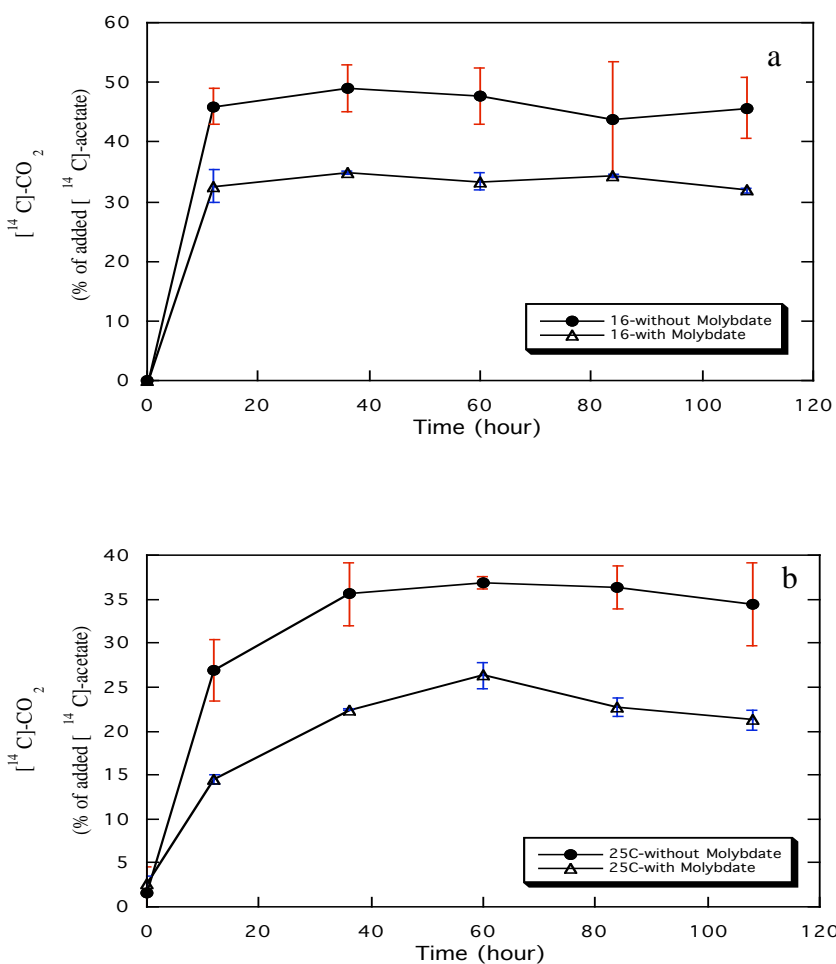


**Figure 5.14 (continued).**

Agarose gels with PCR products generated by the amplification of gDNA using primers specific for: 5b) Lane 1: 500bp DNA Ladder; lanes 2, 3, and 4: DPRB 16S rDNA amplified with CKB-type primers; lanes 5, 6, and 7: DPRB 16S rDNA amplified with RCB-type primers; lanes 8, 9, and 10: DPRB 16S rDNA amplified with PS-type primers (CKB-type, RCB-type, and PS-type primers were developed by Dr Laurie A. Achenbach). Lanes 2, 5, & 8 contain gDNA extracts from an old MPN set containing DPRB; Lanes 3, 6, & 9 are positive controls for each primer set; and, lanes 4, 7, & 10 are negative (no gDNA) controls.

Note: PCR amplification of microbes isolated did not display amplification of the region of 16s rDNA using these set of probes, indicating that these organisms are different from previously isolated DPRB (data not shown).

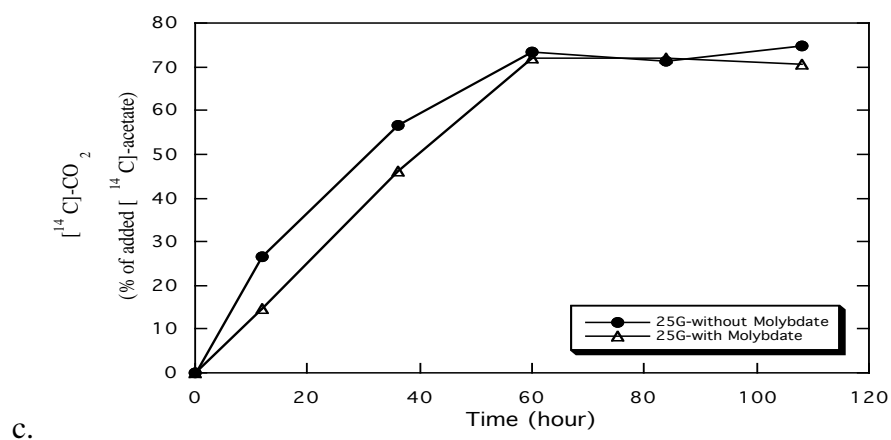




**Figure 5.15**

Terminal Electron Accepting Process determinations for Longhorn, Texas site sediments. Symbols without molybdate are the average of triplicate determinations; symbols with molybdate are the average of duplicate determinations.

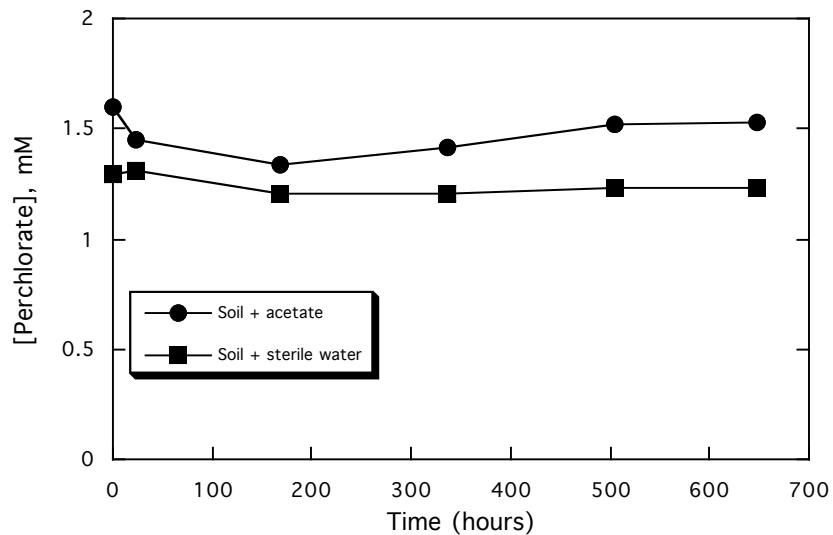
- a. 16= site 16 sediment sample
- b. 25C= site 25 subsurface soil sample



**Figure 5.15 (continued).**

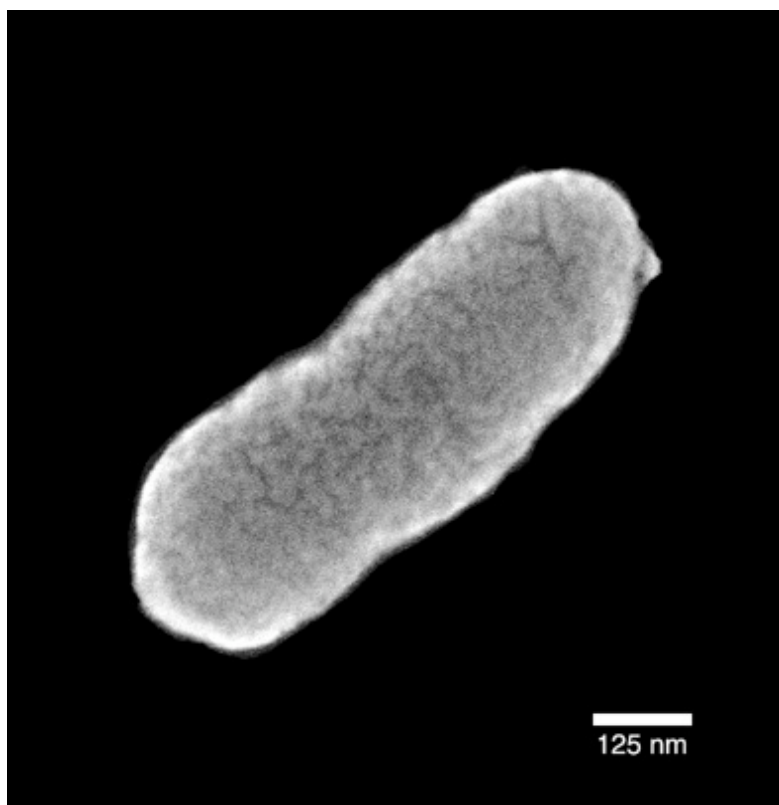
Terminal Electron Accepting Process determinations for Longhorn, Texas site sediments. Symbols without molybdate are the average of triplicate determinations; symbols with molybdate are the average of duplicate determinations.

c. 25G= site 25 sediment sample



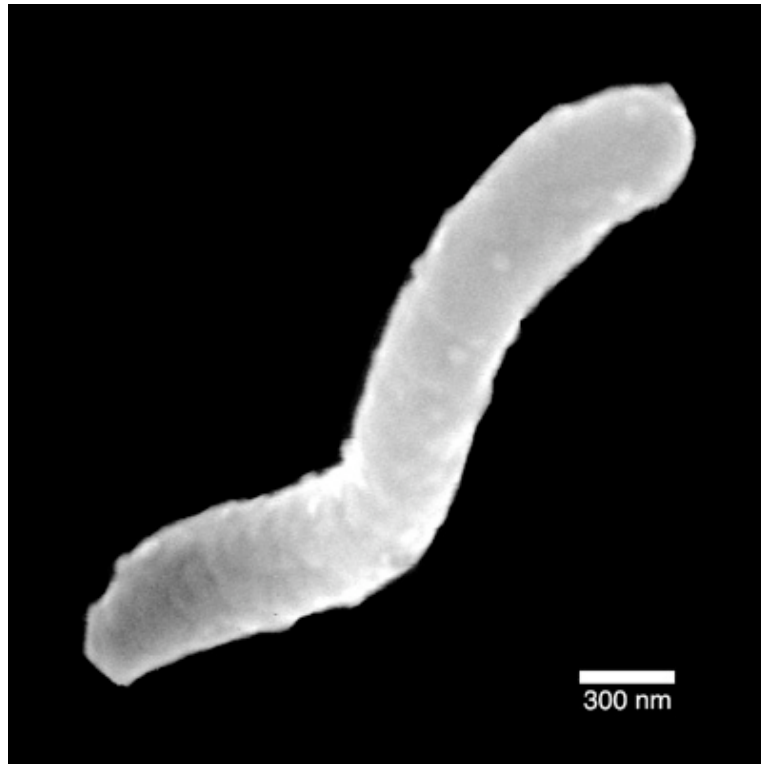
**Figure 5.16**

Perchlorate remediation attempt utilizing soils from Longhorn, Texas. Soils from sites 16, 25C, and 25G were used and all showed the same pattern. Data for site 16 was used as an example. Symbols are the average of triplicate determinations.



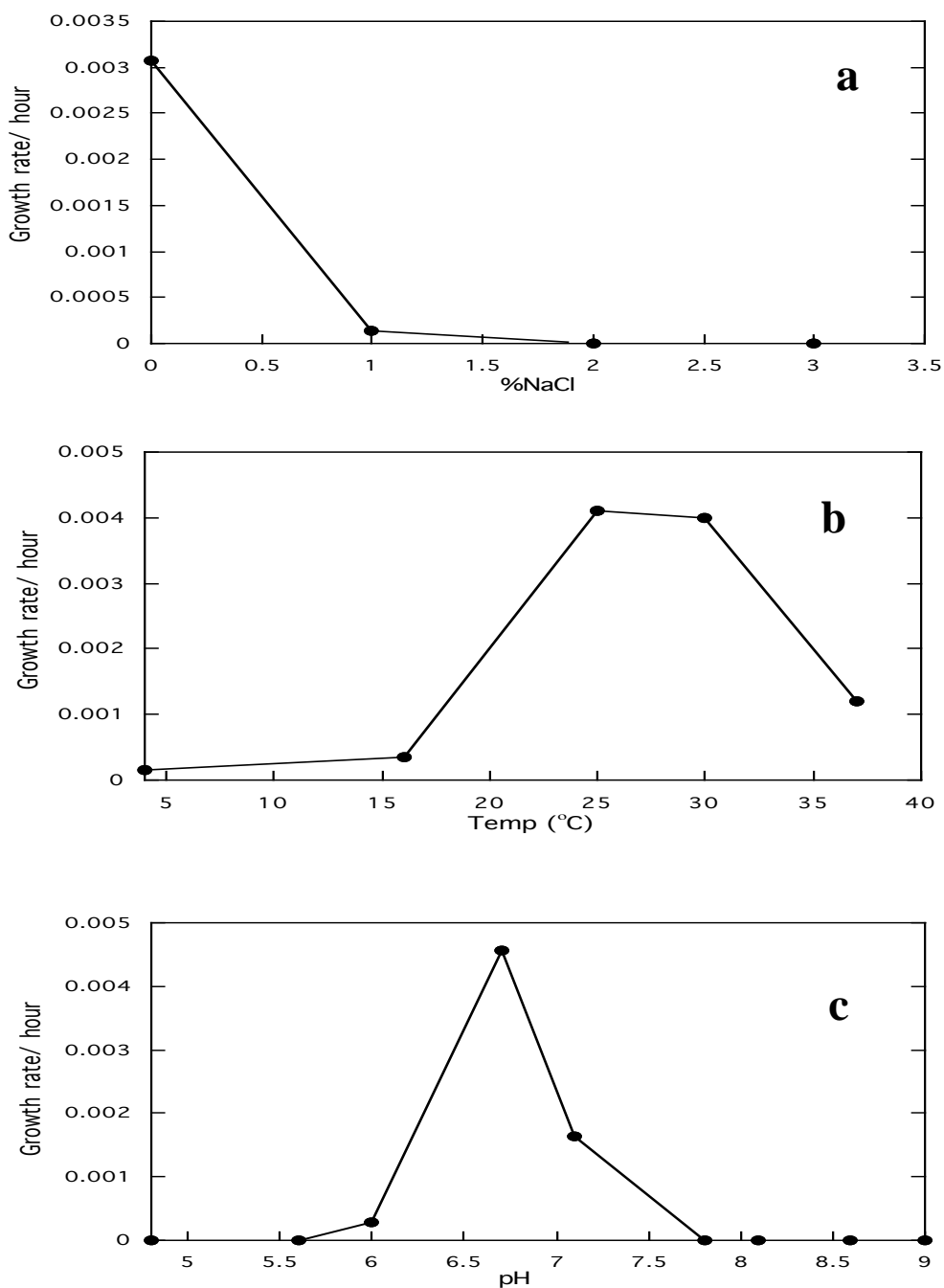
**Figure 5.17**

Scanning electron micrograph of strain LT-1. Strain LT-1 is a Gram negative, motile rod 0.8 microns long by 0.28 microns wide, capable of perchlorate reduction coupled to acetate oxidation.



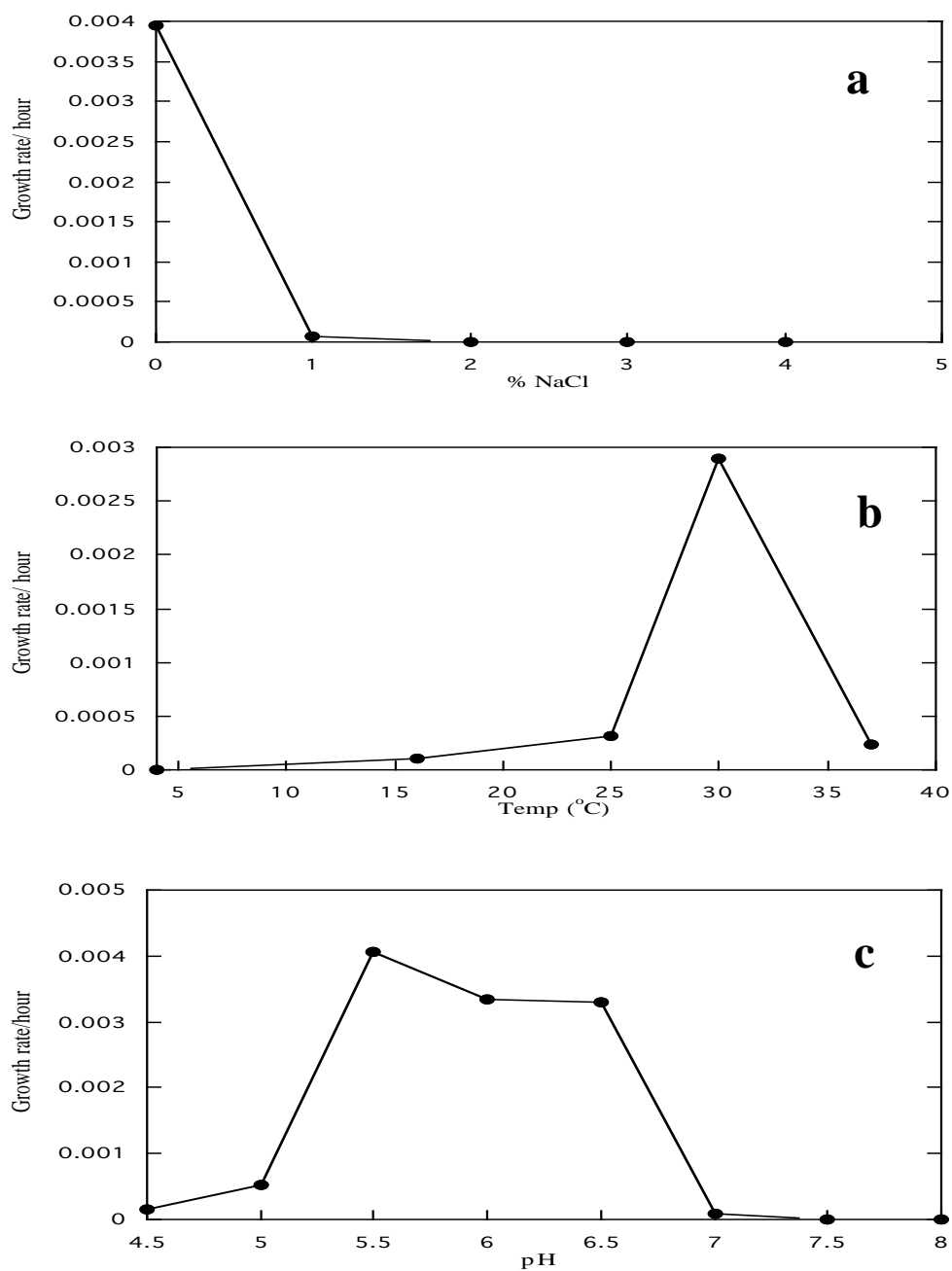
**Figure 5.18**

Scanning electron micrograph of strain AH. Strain AH is a Gram negative, motile curved rod 1 to 1.5 microns long by 0.37 microns wide, capable of perchlorate reduction coupled to acetate oxidation. Under the microscope during cell division, they can appear to be motile spirals, but in fact are two curved rods not yet separated.



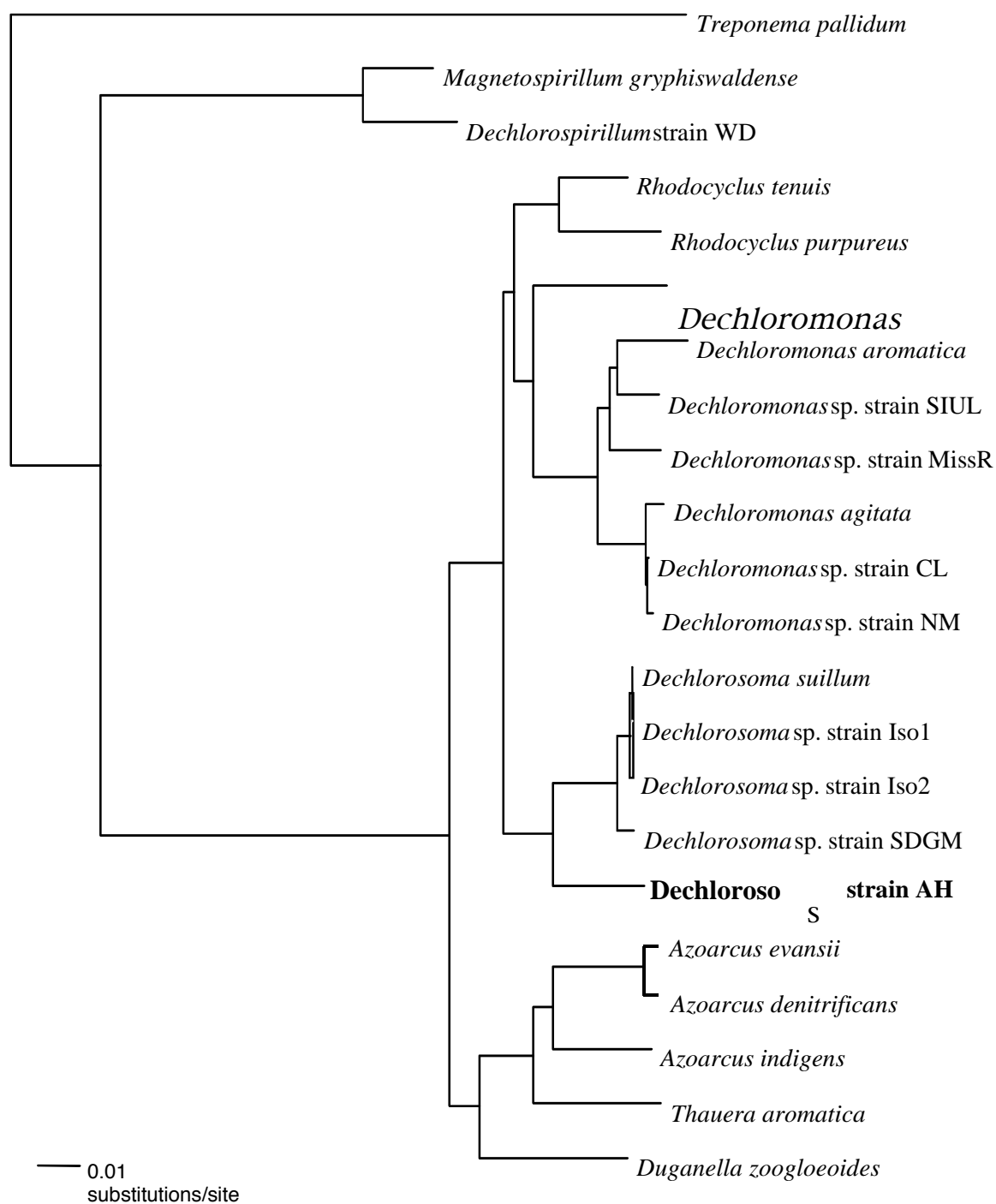
**Figure 5.19**

Growth of strain LT-1 on acetate (10 mM) as the electron donor and perchlorate (10 mM) as the electron acceptor under: a) various sodium chloride concentrations; b) various incubation temperatures, or c) various pH conditions. Symbols are the average of triplicate determinations.



**Figure 5.20**

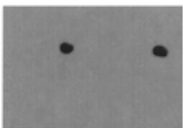
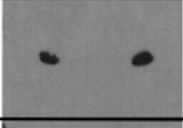
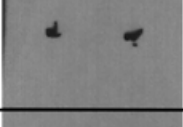
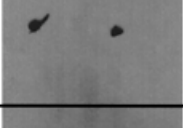
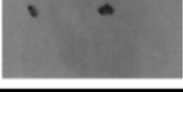
Growth of strain AH on acetate (10 mM) as the electron donor and perchlorate (10 mM) as the electron acceptor under: a) various sodium chloride concentrations; b) various incubation temperatures, or c) various pH conditions. Symbols are the average of triplicate determinations.



**Figure 5.21**

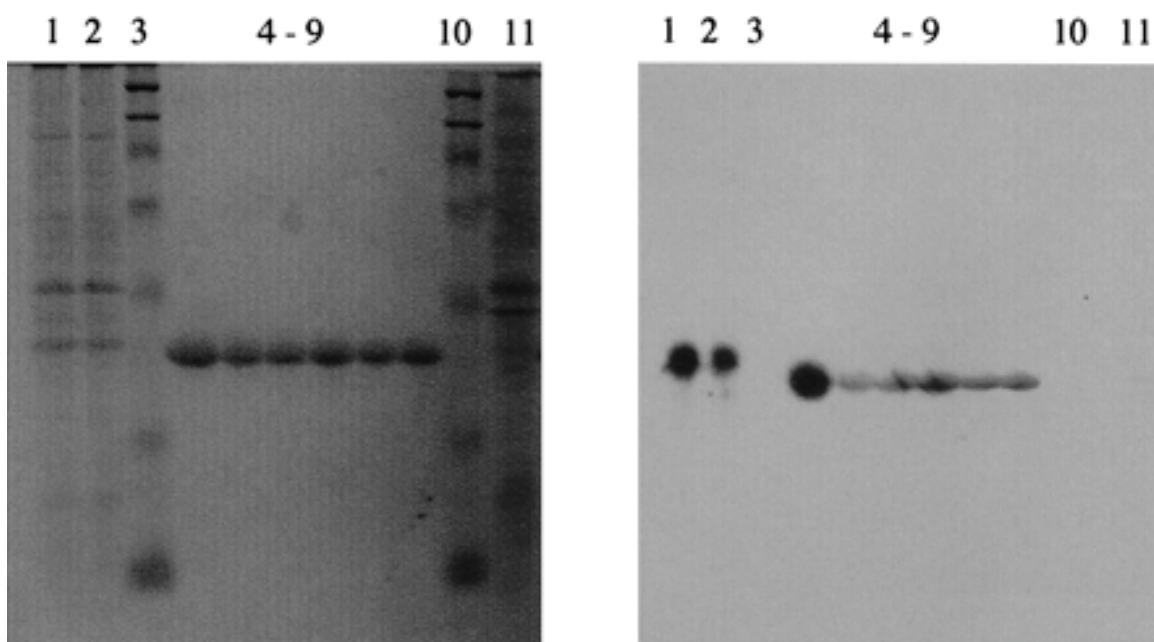
Phylogenetic tree of 16S rDNA data set of 1488 characters showing placement of *Dechloromonas* strain LT-1 and *Dechlorosoma acidophilus* strain AH within the beta subdivision of the Proteobacteria. Only clades recovered in more than 50% of the trees using bootstrap analysis are indicated. Outgroup rooted by *T. pallidum*.



CD-specific IgG dilution	
$5 \times 10^{-4}$	
$1 \times 10^{-5}$	
$2 \times 10^{-5}$	
$4 \times 10^{-5}$	
$1 \times 10^{-6}$	

**Figure 5.22**

Dot blot results of a range of dilutions of the CD-specific IgG. As shown, each sample analysis was performed in duplicate.



**Figure 5.23**

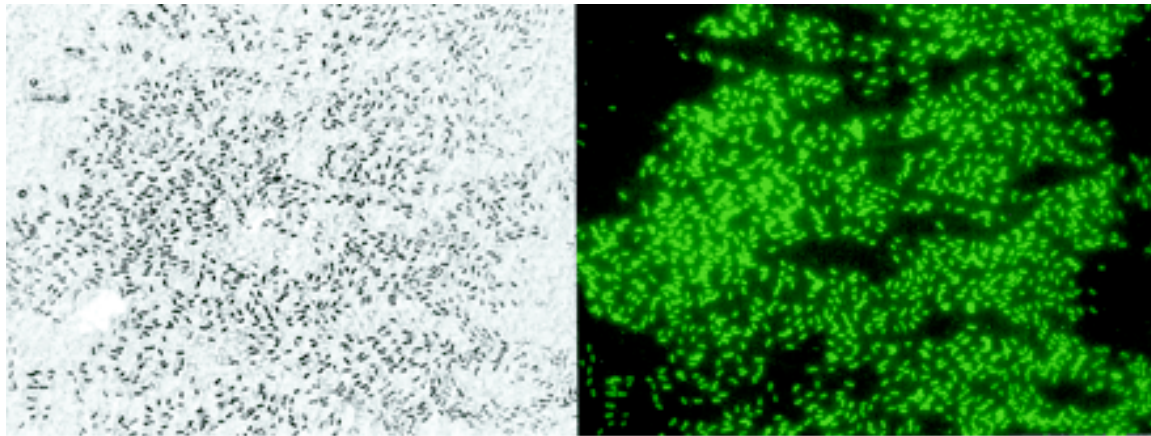
SDS-PAGE gel and Western blot analysis of cell lysates from *D. agitata* and *E. coli* as well as the purified CD enzyme. Lanes 1 and 2, cell lysate from *D. agitata*; lanes 3 and 10, molecular mass marker; lanes 4 through 9, dilutions of purified CD; lane 11, cell lysate from *E. coli*.

Organism	Whole cells	Cell lysate	Proteobacteria subclass
<i>Dechloromonas</i> strain CKB			$\beta$
<i>Dechloromonas</i> strain RCB			$\beta$
<i>Dechlorosoma</i> strain PS			$\beta$
<i>I. dechloratans</i>			$\beta$
<i>Dechlorospirillum</i> strain WD			$\alpha$
<i>Dechloromarinus</i> strain NSS			$\gamma$
<i>Pseudomonas</i> strain PK			$\gamma$
<i>E. coli</i> *			$\gamma$
<i>P. stutzeri</i> *			$\gamma$
<i>R. tenuis</i> *			$\beta$
<i>R. tenuis</i> *, no primary or secondary antibody			$\beta$

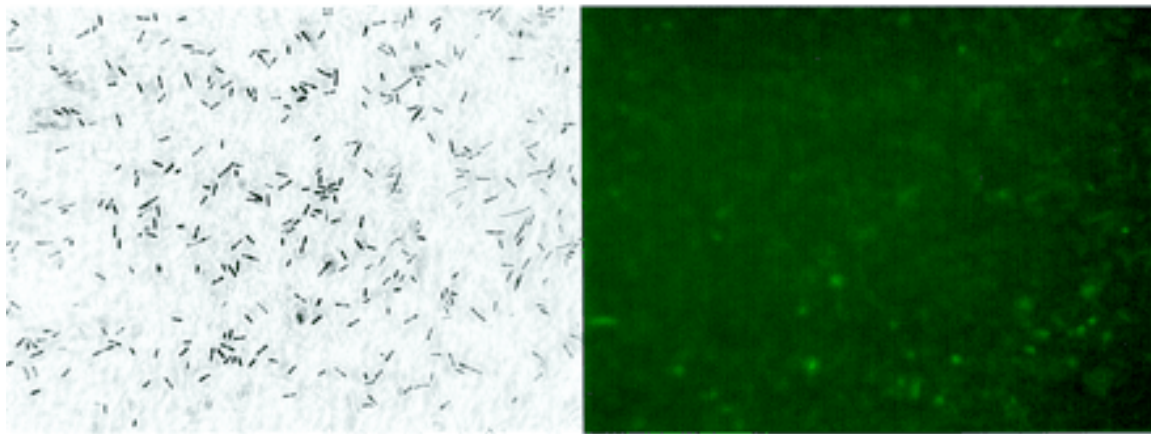
\* these organisms are incapable of reductive (per)chlorate respiration.

**Figure 5.24**

Dot blot results of cell lysates and whole cells of a diverse range of DPRB and their non-(per)chlorate-reducing close relatives. As shown, each sample analysis was performed in duplicate.



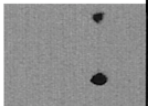
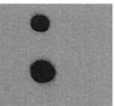


*Dechloromonas agitata*



*Escherichia coli*

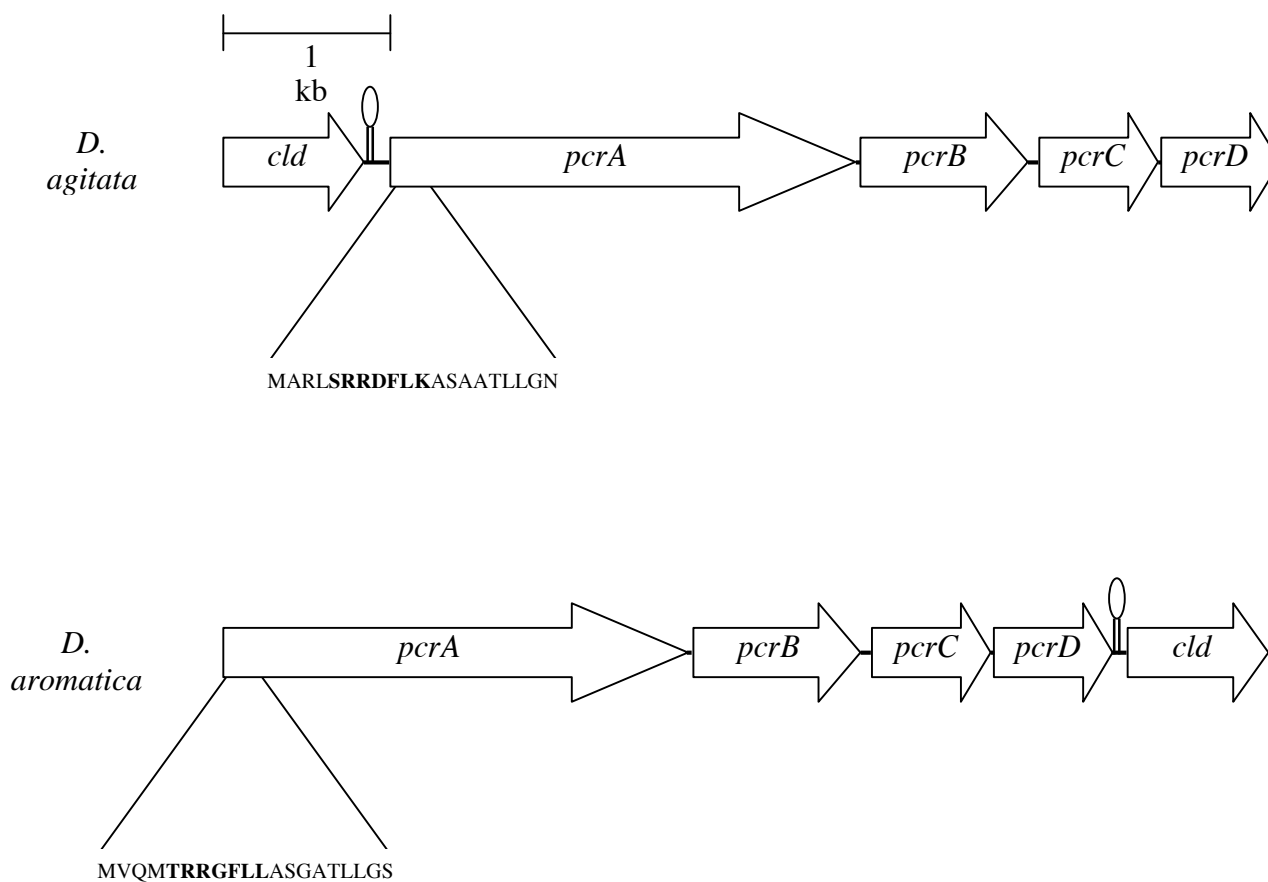
**Figure 5.25**

Phase contrast and immunofluorescence micrographs of whole cells of *D. agitata* and *E. coli* stained with CD-specific IgG primary antibody and fluorescein-labeled goat anti-rabbit IgG secondary antibody.

Organism	Whole cells	Cell lysate
Strain CKB anaerobic		
Strain CKB aerobic		

**Figure 5.26**

Dot blot results of whole cells of *D. agitata* grown aerobically or anaerobically with perchlorate as an electron acceptor. As shown, each sample analysis was performed in duplicate.



**Figure 5.27**

Diagrammatic representation of the *pcrABCD* genes in *D. agitata* and *D. aromatica*. A putative transcriptional terminator is shown downstream of the chlorite dismutase (*cld*) gene in *D. agitata* and downstream of the *pcrD* gene in *D. aromatica*. The N-terminal sequences of PcrA are indicated with the twin arginine motif in bold.

```

1
St.GR1 PcrB -----ANVMKAPRRQLTYVTDxN
D.agit PcrB -----MSNMTKSPKRQLAYVADLNKIGCQCTCTVACKLTWTSGLPGQDYMWRNVETAPGLGYPRNWQSKGGG---YKDGVLQKKGKIPPMID
D.arom PcrB -----MANVMKAPKRQLTYVTDLNKIGCQCTCTVACKLTWTSGLPGQDYMWRNVETAPGLGYPRNWQTKGGG---YKNGELQKKGKIPPMID
T.sele SerB -----MSQRQLAYVFDLNKIGCHTCTMACQQLWTRNDRGFYMYWNNVETSPGKGYPKNNWFOKGGGFD-KDGIKTNGITPTRAD
I.dech C1rB -----MSQRQVAYVFDLNKIGCHTCTMACQQLWTRNDRGREYMYWNNVETSPGKGYPKNNWEGKGGGFD-QEGKLTNGIITPMAD
A.EbN1 EbdB -----MTYVQDGNKSELRAKRLVTVIDLNKIGCQCTCTVACKNIWTKRFGTEHMRWNNVETYPGKGYPRDYERKGGGFL--RGEPPQGVLETLID
R.sulf DdhB -----MVKRQISMVLDLNKIGCQCTCTVACKLQWTRNDRGREYMYWNNVETHPGPGYPRNYEHSGGGFD--EEGALKIGITPSAED
H.mars NarH MSSDQDDQGEEDTLVNIVADGVDHQVAMVMDLNKIGCQCTCTIACKNLWTEDEGSEYMYWNNVETKPGEGYPRGWENS GGGWKSGEHKEQPGEIPDEED

101
D.agit PcrB YGVPFEDYAGR-LFEGKKERARPS-----TPRYAPNWDEDOGAGEYPNNSFFVLPVMCNHCAKPALEACPNEAIYKREQDGLVVIHQKCKGAQA
D.arom PcrB YGIPFEDYAGR-LFEGKKERVPS-----TPRSAPNWDEDOGAGEYPNNSFFVLPVMCNHCTKPALEACPNEAIYKREQDGLVVIHQKCKGAQA
T.sele SerB YGGTWNYNLET-LVEGKSNQVVE-----KETWGPNWDEDEGKGEFPNNHYFYLPRI CNHCSNPAFLAACPTKAIYKREDGLVVDQSRCKGYRY
I.dech C1rB YGGRIGDFNLNEVLLEGKADQVVE-----KATWGPNWDEDEGKGEFPNNHSFYLPRI CNHCSNPAFLAACPTKAIYKREDGLVVDQTRCRGYRY
A.EbN1 EbdB GSDDEQENIKEY-FYEGKQTVIIFITSKSTGKDFAWGYNWDEDOGGGKWPFPFFYLARMCNHCTNPAFLAACPTKAIYKREDNGIIVLDQERCKGHRI
R.sulf DdhB YGTPWFYNYFRA-LMTGTDPWLRPNV-----KPTWGANWNRDRGRCEYPNSYFYVLPRI CNHCANPGCLAACARNAIYKREQDGLVVDQRRCRGYRY
H.mars NarH YGRAWEENHEEI-MYEGSDEPLRPD-----GAEWGPNWDEDOGAGEYPNSYFYVLPRI CNHCTHESLVEACPRSAIYKREQDGLVVDQRRCRGYRY

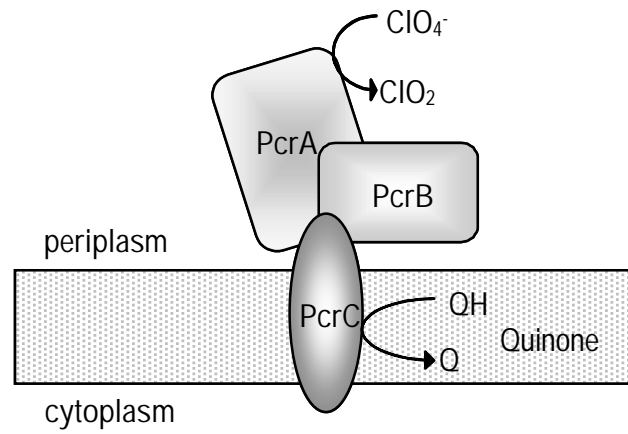
201
D.agit PcrB CIGSCPYAKPYFNAQVNKANKIGCFPRIEK-GV-----APACVAEGAGRAMHVGFIDDESSVFKLVKRFQVALPLHPEYGTENPVFYVPPVL-GPRVE
D.arom PcrB CIGSCPYAKPYFNPVANKANKIGCFPRIEQ-GV-----APGCVACQVGRAMHVGFIDDTNSSVHKLIRLYKVALPLHPEFGTEPNVYVPPVL-GPRME
T.sele SerB CVKACPYGKMYFNLQGTSEKIGCYPRVEK-GE-----APACVVKQSGRIREFWGYRDDKDGFIYKLVQWQVALPLHAEYGTENPVFYVPPMNTTTPPF
I.dech C1rB CVKACPYGKMYFNLQGTSEKIGCYPRVEK-GE-----APACVVKQSGRIREFWGYRDDKNGPIYKLVQWQVALPLHAEYGTENPVFYVPPMNTTTPPF
A.EbN1 EbdB CVEACPYKAIYFNPVSQTSEKICILYPRIEK-GI-----ANACNRQCPGRVRAFGYLDGTTSHVHKLIVKKWQVALPLHAEYGTGPNIIYVPPM--GARGF
R.sulf DdhB CITACPYKKVYFNEQISKAKECIFYPRIEK-GL-----PTACAKQCVGRIRFIFYLDDEAGFVHLVVERYKVAIPLHPEWGTKPSVEFYVPPVL--APPRI
H.mars NarH CVEGCPYKKVYNTVSKSEKICIFYPRIEGEGPDGETFAPACAECPQQLRLVGLFDDEEGPIHKLIVNEYEVALPLHPEFRTQPNVYIIPF--APGQH

301
D.agit PcrB MPNGEHTADPKISMTQLEQLFGKQVREVLKTLQAEREKKIKNQPSLEMDILIGRRSADMMISPMT-----
D.arom PcrB LPNGELSTDPKIPLAQLEGLFGKQVRDVLAILQTEREKKMKGLASDLMVDLIGRRSADMMISPLT-----
T.sele SerB EEDGRLGDKRIPIDLEALFGPGVKQALATLGGEMAKRRKAQASELTDILIGYTNKDRYGI-----
I.dech C1rB EEDGRLGDKRIPIDLEALFGPGVKQALATLGGEMAKRRKAQASELTDILIGFTNKDRYGV-----
A.EbN1 EbdB GEDGETIDTRIPLDVLEGLFGPEVKRVLAVLITERENNRAGRGSSELMDDLISKKWSDRFGGTNDPLTQS
R.sulf DdhB GDDGEPTETRVPLAYLKLFGFAVVALETLLKTERAKKQSGAESELMDTLIGYRHPMEFKLS-----
H.mars NarH TEDGETVDIDRIPRQYLRDLFGDGVQALDTIERERQARQGEDSELMELLQDKNPAKQYRLEVFDDE---
3/1

```

**Figure 5.28**

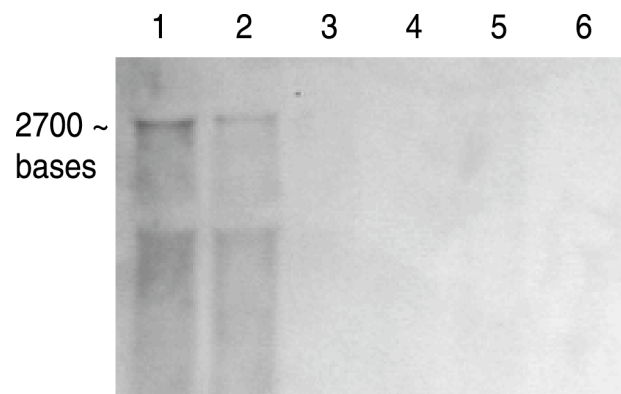
Amino acid alignment of the  $\beta$  subunit of perchlorate reductase (PcrB) from strain GR-1 (N-terminal sequence only), *D. agitata* and *D. aromatica*, selenate reductase B (SerB) from *T. selenatis*, chlorate reductase (DPRB) from *I. dechloratans*, ethylbenzene dehydrogenase B (EbdB) from *Azoarcus* sp. strain EbN1, dimethyl sulphide dehydrogenase B (DdhB) from *R. sulfidophilum*, and nitrate reductase H (NarH) from *H. marismortui*. Light shading indicates amino acids identical to perchlorate reductase. Darkly shaded residues indicate conserved cysteine clusters.



**Figure 5.29**

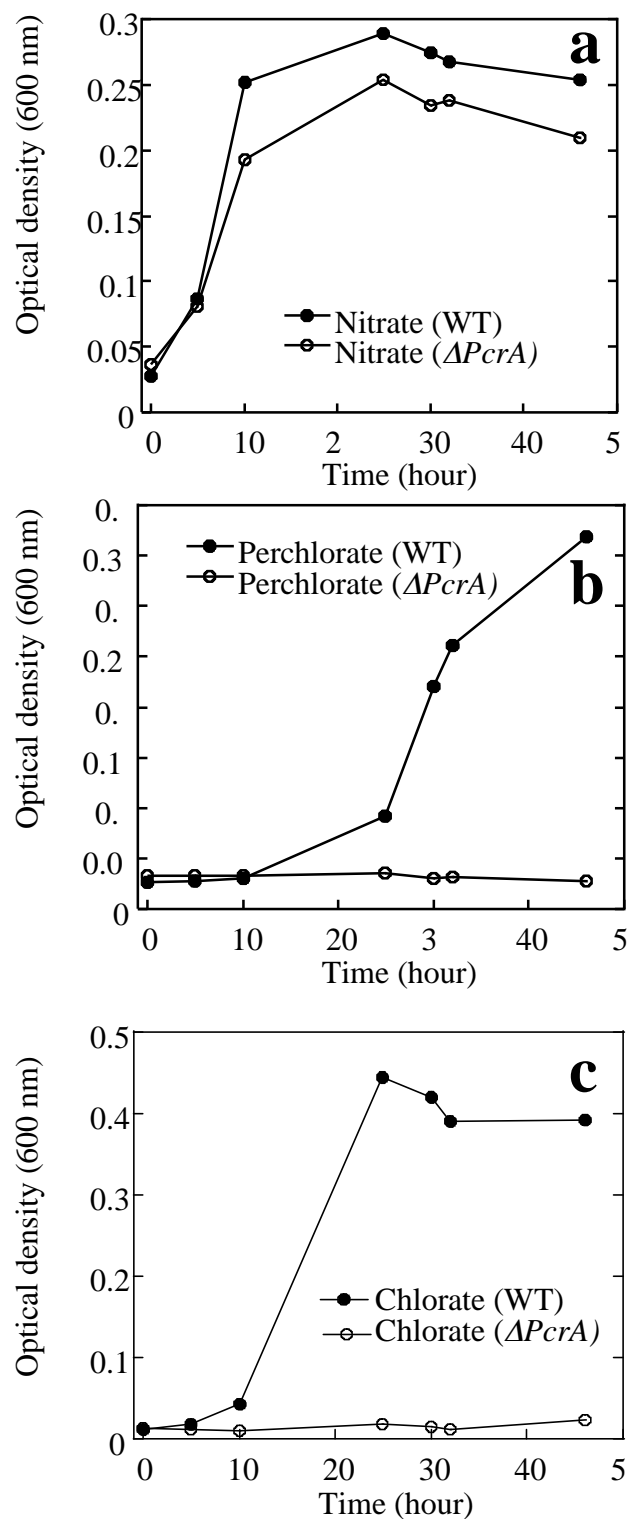
Predicted model of electron transfer during (per)chlorate reduction. Electrons from a quinone pool are transferred from the membrane via PcrC to the PcrAB reductase.





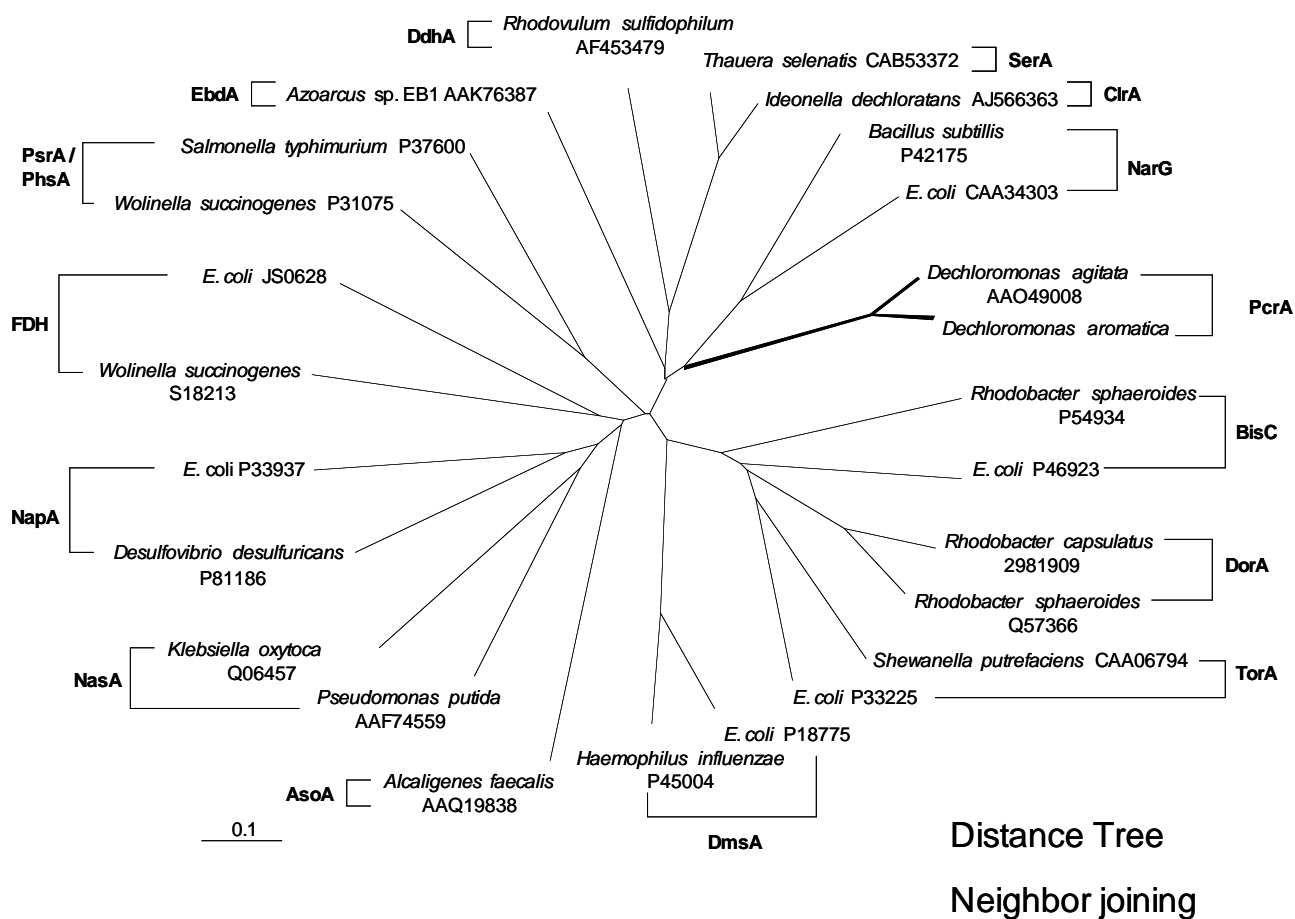
**Figure 5.30**

Northern blot of *D. agitata* RNA hybridized with a *pcrA* probe. Growth conditions: Lane 1) perchlorate, Lane 2) chlorate, Lane 3) aerobic, 4) aerobic supplemented with 1 mM perchlorate, 5) aerobic supplemented with 1 mM chlorate, 6) aerobic supplemented with 1 mM nitrate.



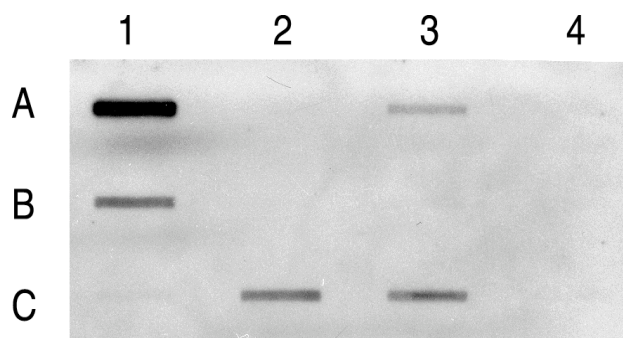
**Figure 5.31**

Anaerobic growth of the wild type *D. aromatica* and the  $\Delta PcrA$  deletion mutant with (a) nitrate, (b) perchlorate, and (c) chlorate as the sole electron acceptors. The results depicted are the average of duplicate incubations.



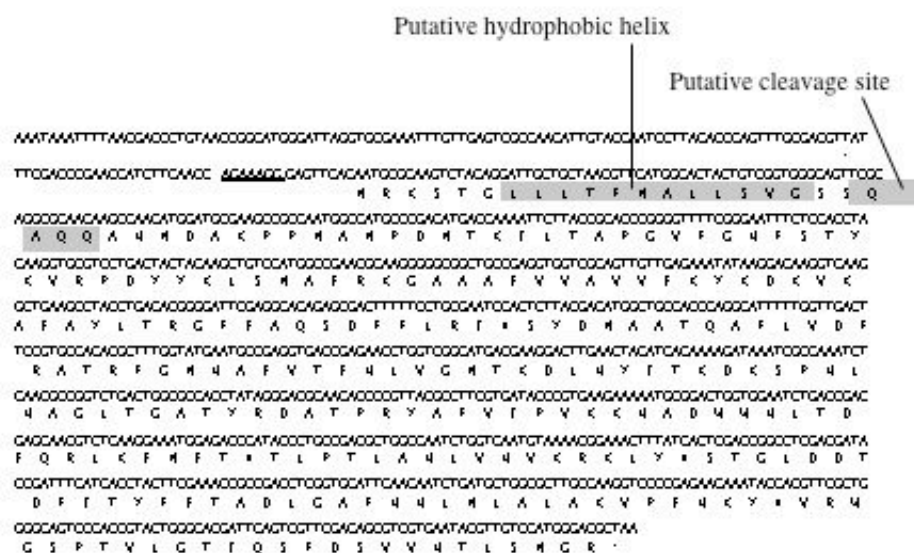
**Figure 5.32**

Unrooted neighbor-joining tree indicating the evolutionary distances within the DMSO reductase family of molybdoenzymes. GenBank accession numbers are indicated.



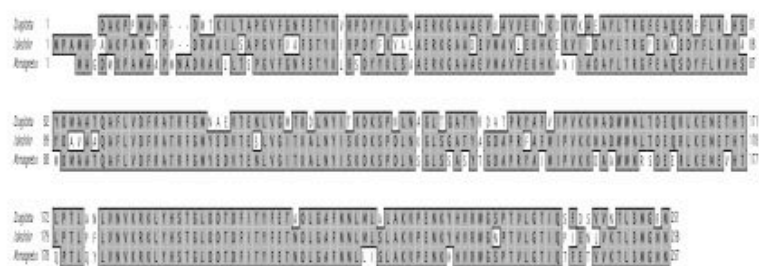
**Figure 5.33**

Slot-blot hybridization of gDNAs from DPRB and non-perchlorate reducing close relatives using the *D. agitata pcrA* probe. Organisms capable of perchlorate reduction are underlined; those capable of only chlorate reduction are double-underlined. Row A (left to right): 1) *Dechloromonas agitata*, 2) *Rhodocyclus tenuis*, 3) *Dechloromonas aromatica*, 4) *Dechloromonas* strain JJ. Row B: 1) *Dechlorospirillum anomalous* strain WD, 2) *Magnetospirillum magnetotacticum*, 3) *Pseudomonas* strain PK, 4) *Pseudomonas stutzeri*. Row C: 1) *Dechloromarinus chlorophilus* strain NSS, 2) *Azospira suillum*, 3) *Dechloromonas* strain LT-1, 4) *Ideonella dechloratans*



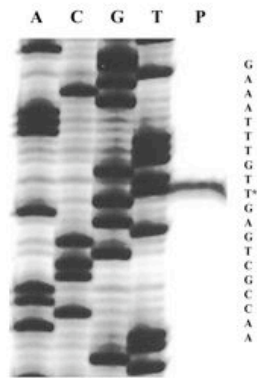
**Figure 5.34**

Nucleotide and predicted amino acid sequence of the *D. agitata* chlorite dismutase gene. The asterisk indicates the transcription start site and the putative -10 promoter region immediately upstream of the start is double-underlined. The ribosome binding site is underlined. The putative helix and cleavage sites in the leader peptide are indicated.



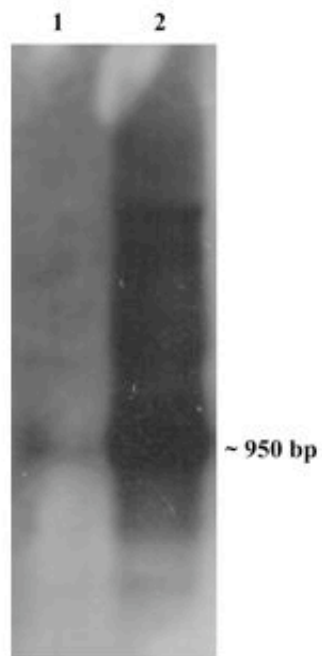
**Figure 5.35**

Amino acid sequence alignment of the mature *D. agitata* and putative *I. dechloratans* and *M. magnetotacticum* chlorite dismutase proteins.



**Figure 5.36**

Primer extension using RNA from a perchlorate-grown culture of *D. agitata*. The sequencing ladder was generated using the same primer as the primer extension reaction.

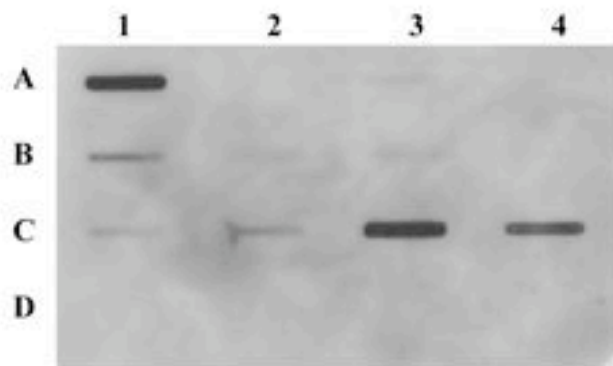


**Figure 5.37**

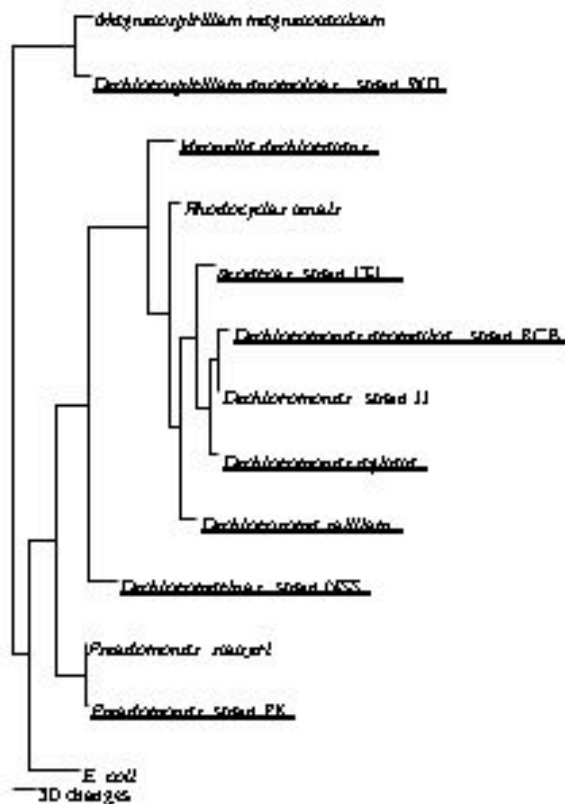
Northern blot of *D. agitata* RNA hybridized with the *D. agitata* probe to the 3'-end of the chlorite dismutase gene. Lane 1) aerobic growth and Lane 2) anaerobic growth with perchlorate as the electron acceptor.



Panel A



Panel B



**Figure 5.38**

Panel A) Slot-blot hybridization of gDNAs from DPRB and non-perchlorate reducing close relatives using the *D. agitata* probe to the 3'-end of the chlorite dismutase gene. Organisms capable of perchlorate or chlorate reduction are underlined. Row1 (left to right): *Dechloromonas agitata*, *Rhodocyclus tenuis*, *Dechloromonas aromatica* strain RCB, *Dechloromonas* strain JJ. Row 2: *Dechlorospirillum anomalous* strain WD, *Magnetospirillum magnetotacticum*, *Pseudomonas* strain PK, *Pseudomonas stutzeri*. Row 3: *Dechloromonas* strain NSS, *Dechlorosoma suillum*, *Azoarcus* strain LT-1, *Ideonella dechloratans*. Row 4: *E. coli*. Panel B) Phylogenetic tree of the organisms used in the slot-blot hybridization based on 16S rDNA sequences.

## Appendix B (Tables)

**Table 4.1**

Descriptions of samples used in stable isotope analysis

<b>Weedkiller Samples</b>	
<b>Manufacturer</b>	<b>Size range</b>
Doff	>1 $\mu$ m, <2 $\mu$ m
Doff	>250 $\mu$ m, <500 $\mu$ m
Doff	>63 $\mu$ m, <125 $\mu$ m
Gem	>1 $\mu$ m, <2 $\mu$ m
Gem	>250 $\mu$ m, <500 $\mu$ m
Gem	>63 $\mu$ m, <125 $\mu$ m
<b>Flashpowder samples</b>	
<b>Sample number</b>	<b>Details</b>
1	White flash powder
2	Amber flash powder
3	Blind flash powder
4	Red flash powder
5	Thunderflash

**Table 4.2**

Primer sets used for amplifying the CD gene from different DPRB.

Target site	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Product size
<i>I. dechloratans</i> <i>cd gene</i>	ICD741F-TATCTCCAAGGA CAAGTCGC	ICD1140R-TCAATTGCCC AT CGACAGCGT	399 bp
<i>D. agitata</i> middle <i>cd gene</i>	CD150F-CGCAGTTGTTGA GAAATATA	CD581R-GTAGGTGATGA AATCGGTAT	431 bp
<i>D. agitata</i> 3' end of <i>cd gene</i>	CD364F-AAAAGATAAATC GCCAAATC	CD756R-TTAGCGTCCCA TGGACAACG	392 bp
<i>D. agitata</i> 5' end of <i>cd gene</i>	CD107RC-ACAGTAGTGCC ATGAACGTT		Primer exten.

**Table 5.1**

Most probable number counts of acetate-oxidizing, chlorate reducers in different environments.

Environment	Counts (cells per gram)
Swine waste lagoon	$2.40 \pm 1.74 \times 10^6$
Pristine aquatic sediment	$4.62 \pm 1.75 \times 10^3$
Mississippi river sediment	$4.27 \pm 2.14 \times 10^3$
Pristine soil	$2.31 \pm 1.33 \times 10^3$
Gold mine drainage sediment	$4.27 \pm 2.14 \times 10^3$
Petroleum contaminated soil	$9.33 \pm 4.17 \times 10^3$
Pohic Bay	$1.49 \pm 0.60 \times 10^4$
Florida Swamp	$2.31 \pm 1.33 \times 10^4$

**Table 5.2**

Compounds used as electron donors by different (per)chlorate-reducing isolates with chlorate (10 mM) as the electron acceptor.

Electron donor	Concentration	(per)chlorate-reducing isolate							
		NM	CL	MSSR	PS	ISO1	ISO2	SDGM	WD
Acetate	10 mM	+	+	+	+	+	+	+	+
Propionate	10 mM	+	+	+	+	+	+	+	+
Isobutyrate	10 mM	+	+	+	+	+	+	+	+
Butyrate	10 mM	+	+	+	+	+	+	+	+
Valerate	10 mM	+	+	+	+	+	+	+	+
Formate	10 mM	-	-	+	-	-	-	-	-
Methanol	5 mM	-	-	-	-	-	-	-	-
Ethanol	5 mM	-	-	+	+	+	+	+	-
Catechol	1 mM	-	-	-	-	-	-	-	-
Glycerol	5 mM	-	-	-	-	-	-	-	-
Benzoate	0.5 mM	-	-	-	-	-	-	-	-
Pyruvate	5 mM	+	nd	nd	+	nd	nd	nd	nd
Citrate	5 mM	-	-	-	-	-	-	-	-
Succinate	1 mM	+	+	-	+	-	-	-	+
Lactate	10 mM	+	+	+	+	+	+	+	+
Glucose	10 mM	-	-	-	-	-	-	-	-
Casamino Acids	1g/L	+	-	+	+	+	+	+	+
Fumarate	5 mM	+	+	+	+	+	+	+	+
Malate	1 mM	+	+	+	+	+	+	+	+
Hydrogen	101 KPa	-	-	-	-	-	-	-	-
Fe(II)	5 mM	nd	nd	nd	+	nd	nd	nd	+

+, utilization of electron donor; -, no utilization; nd, not determined

**Table 5.3**

Compounds used as electron acceptors by (per)chlorate-reducing isolates with acetate (10 mM) as the electron donor.

Electron acceptor	Concentration	<b>(per)chlorate-reducing isolate</b>							
		NM	CL	MSSR	PS	ISO1	ISO2	SDGM	WD
Chlorate	10 mM	+	+	+	+	+	+	+	+
Nitrate	5 mM	-	-	+	+	+	+	+	+
Sulfate	10 mM	-	-	-	-	-	-	-	-
Selenate	2 mM	-	-	-	-	-	-	-	-
Fumarate	25 mM	-	-	-	-	-	-	-	-
Malate	5 mM	-	-	-	-	-	-	-	-
Mn (IV)	2 mM	-	-	-	-	-	-	-	-
Fe(III)	10 mM	-	-	-	-	-	-	-	-
O <sub>2</sub>	101 kPa	+	+	+	+	+	+	+	+
AQDS	5mM	-	-	-	-	-	-	-	-
Fermentation		-	-	-	-	-	-	-	-

+, utilization of electron acceptor; -, no utilization

**Table 5.4**

Results obtained of chlorine istable sotopic analyses of flashpowder perchlorate

Sample	37Cl, ‰							Mean	1
White flashpowder	-0.15	-0.15	+0.03	-0.03	0.00	+0.22	-0.02	-0.05	+0.08
Amber flashpowder	+0.21	+0.20	+0.67	+0.16	+0.28	+0.34	+0.16	+0.23	+0.19
Blind flashpowder	-0.15	+0.03	-0.19	-0.01	+0.05	+0.09	+0.08	-0.01	+0.09
Red flashpowder	+0.24	+0.02	+0.20	+0.39	+0.11	+0.25	+0.17	+0.20	+0.13
Thunderflash	+0.09	-0.07	+0.07	+0.02	+0.20	+0.09	-0.10	+0.04	+0.11

**Table 5.5**

Geochemical analysis of soils collected from San Nicolas Island, California.

Site-Depth	ClO <sub>4</sub> <sup>-</sup> (μM)	SO <sub>4</sub> <sup>2-</sup> (mM)	Cl <sup>-</sup> (mM)	Fe <sup>2+</sup> (mM)	Fe <sup>3+</sup> (mM)	NO <sub>3</sub> <sup>-</sup> (mM)	pH
<b>“1000 Springs”</b>							
Pristine-3 ft	0.68	2.88	42.82	5.28	9.85	0	8.9
Pristine-5.5 ft	0	2.45	54.41	30.70	11.17	0	8.8
Pristine-8 ft	0	1.39	32.05	32.32	22.69	0	9.0
<b>“Explosive Ordinance Disposal”</b>							
Medium-3 ft	3.75	0.24	3.81	2.34	5.66	0.007	8.6
Medium-6 ft	1.17	0.11	0.93	1.67	5.74	0	8.4
Medium-9 ft	0.78	0.19	2.72	2.41	9.81	0	8.7
<b>“Drainage Channel from EOD”</b>							
Heavy-3 ft	27.69	0.47	41.71	4.65	1.69	0	8.0
Heavy-6 ft	10.76	0.26	12.26	4.24	5.04	0	8.7
Heavy-9 ft	2.86	0.14	1.90	11.88	1.97	0	8.4



**Table 5.6**

Most probable number (MPN) counts of acetate-oxidizing, perchlorate-reducing bacteria from San Nicolas Island, California sediments.

<u>Site-Depth</u>	<u>Cells/gram of sediment or per ml of groundwater</u>
<b>“1000 Springs”</b>	
Pristine-3 ft	No growth
Pristine-5.5 ft	$2.40 \pm 1.74 \times 10^5$
Pristine-8 ft	$2.40 \pm 1.74 \times 10^5$
<b>“Explosive Ordinance Disposal”</b>	
Medium-3 ft	$7.49 \pm 3.35 \times 10^4$
Medium-6 ft	$7.49 \pm 3.35 \times 10^4$
Medium-9 ft	No growth
<b>“Drainage Channel from EOD”</b>	
Heavy-3 ft	No growth
Heavy-6 ft	No growth
Heavy-9 ft	No growth

**Table 5.7**

PCR results for highest dilution MPN from San Nicolas Island, California. Universal (8F and 1525R) primers were used on bacterial 16S rDNA unless otherwise stated.

Site-Depth	Universal	WD	CKB	RCB	PS
<b>“1000 Springs”</b>					
Pristine-3 ft	No Growth				
Pristine-5.5 ft	+(8F&530R)	-	-	-	-
Pristine-8 ft	+(8F&530R)	-	-	-	-
<b>“Explosive Ordinance Disposal”</b>					
Medium-3 ft	+	-	-	-	-
Medium-6 ft	+	-	-	-	-
Medium-9 ft	No Growth				
<b>“Drainage Channel from EOD”</b>					
Heavy-3 ft	No Growth				
Heavy-6 ft	No Growth				
Heavy-9 ft	No Growth				

**Table 5.8**

PCR results for soil extractions from San Nicolas Island, California.

Site-Depth	Universal	WD	CKB	RCB	PS
<b>“1000 Springs”</b>					
Pristine-3 ft	+	–	–	–	–
Pristine-5.5 ft	–	–	–	–	–
Pristine-8 ft	+	–	–	–	–
<b>“Explosive Ordinance Disposal”</b>					
Medium-3 ft	+	–	–	–	–
Medium-6 ft	+	–	–	–	–
Medium-9 ft	–	–	–	–	–
<b>“Drainage Channel from EOD”</b>					
Heavy-3 ft	+	–	–	–	–
Heavy-6 ft	+	–	–	–	–
Heavy-9 ft	+	–	–	–	–

**Table 5.9**

Geochemical analysis of soils and groundwater from Longhorn, Texas.

Site	ClO <sub>4</sub> <sup>-</sup> (μM)	SO <sub>4</sub> <sup>2-</sup> (mM)	Cl <sup>-</sup> (mM)	Fe <sup>2+</sup> (mM)	Fe <sup>3+</sup> (mM)	NO <sub>3</sub> <sup>-</sup> (mM)	pH
16 (Sediment)	19.78	29.61	40.51	0	1.25	0.06	6.6
16 (Groundwater)	2.21	2.96	6.57	1.86	9.45	0	6.4
25C (Subsurface soil)	4.00	0.15	0.29	4.10	28.16	0.06	4.2
25G (Sediment)	792.5	3.94	5.80	0	1.34	0.09	5.6
25G (Groundwater)	89.69	0.29	0.34	2.53	11.00	0.06	6.5

**Table 5.10**

Most probable number (MPN) counts of acetate-oxidizing, perchlorate-reducing bacteria from Longhorn, Texas sediments and ground water.

Site	Cells/gram of sediment or per ml of groundwater
16 (Sediment)	$2.40 \pm 1.74 \times 10^6$
16 (Ground Water)	$2.15 \pm 0.81 \times 10^6$
25C (Subsurface soil)	$9.33 \pm 4.17 \times 10^5$
25G (Sediment)	$2.40 \pm 1.74 \times 10^5$
25G (Groundwater)	$1.12 \pm 0.64 \times 10^3$

**Table 5.11**

PCR results for highest dilution MPN from Longhorn, Texas.

Site	Universal	WD	CKB	RCB	PS
16 (Sediment)	+	–	–	–	–
16 (Groundwater)	+	–	–	–	–
25C (Subsurface soil)	+	–	–	–	–
25G (Sediment)	+	–	–	–	–
25G (Groundwater)	+	–	–	–	–

**Table 5.12**

PCR results for soil extractions from Longhorn, Texas.

Site	Universal	WD	CKB	RCB	PS
16 (Sediment)	+	-	-	-	-
16 (Ground Water)	+	-	-	-	-
25C (Subsurface soil)	+	-	-	-	-
25G (Sediment)	+	-	-	-	-
25G (Ground Water)	+	-	-	-	-

**Table 5.13**

Electron donors tested for growth of the new DPRB isolates strains LT-1 and AH with 10 mM perchlorate as the electron acceptor.

Electron donor	Concentration (mM)	Strain LT-1	Strain AH
Acetate	10	+	+
Formate	10	–	–
Propionate	5	+	+
Lactate	10	–	+
Succinate	5	+	+
Fumarate	5	+	+
Pyruvate	10	+	+
Malate	10	+	+
Citrate	10	–	–
Palmitate	1	+	–
Butyrate	5	+	–
Valerate	5	+	–
Isovalerate	5	+	–
Hexanoic Acid	5	+	–
Heptanoic Acid	5	–	–
Casamino Acids	1 gm/ L	+	+
Yeast Extract	1 gm/ L	+	+
Fe(II) Chloride (w 0.1mM Acetate)	10	–	–
Hydrogen (w 0.1 mM Acetate)	101KPa	+	–
Thiosulfate (w 0.1 mM Acetate)	10	–	–
Fumarate <u>only</u>	10	–	–
Fermentation (glucose, casamino acids, and YE)		–	–
Ethanol	10	–	+
Methanol	10	–	–
Glycerol	10	–	–



**TABLE 5.14**Blast analysis of the *pcrABCD* translation products.

<b>GenBank Blast Hits</b>		<b>% AA Identity</b>
<b>gene</b>		
<i>pcrA</i>	CAD22069 <i>Haloarcula marismortui</i> - nitrate reductase $\alpha$ -subunit (NarG)	41
	CAF21906 <i>Haloferax mediterranei</i> - nitrate reductase $\alpha$ -subunit (NarG)	40
	AAN46632 <i>Rhodovulum sulfidophilum</i> - dimethyl sulphide dehydrogenase $\alpha$ -subunit (DdhA)	34
	Q9S1H0 <i>Thauera selenatis</i> - selenate reductase $\alpha$ -subunit (SerA)	33
	P60068 <i>Ideonella dechloratans</i> - chlorate reductase $\alpha$ -subunit (ClrA)	33
<i>pcrB</i>	Q9S1G9 <i>Thauera selenatis</i> - selenate reductase $\beta$ -subunit (SerB)	54
	P60069 <i>Ideonella dechloratans</i> - chlorate reductase $\beta$ -subunit (DPRB)	54
	CAD58340 <i>Azoarcus</i> sp. strain EbN1- ethylbenzene dehydrogenase $\beta$ -subunit (EbdB)	53
	AAN46633 <i>Rhodovulum sulfidophilum</i> - dimethyl sulphide dehydrogenase $\beta$ -subunit (DdhB)	52
<i>pcrC</i>	NP_842334 <i>Nitrosomonas europaea</i> - cytochrome <i>c</i> -554 precursor	49
<i>pcrD</i>	Q9S1G8 <i>Thauera selenatis</i> - selenate reductase (SerD)	35
	AAN46634 <i>Rhodovulum sulfidophilum</i> - dimethyl sulphide dehydrogenase $\delta$ -subunit (DdhD)	29
	CAD58338 <i>Azoarcus</i> sp. strain EbN1- ethylbenzene dehydrogenase $\delta$ -subunit (EbdD)	25
	CAD22073 <i>Haloarcula marismortui</i> - nitrate reductase molybdenum chaperone (NarJ)	25

## **Appendix C**

### **Accomplishments to date since inception of project CU-1162**

#### **Patents pending**

- 1 “Bio-Sensor for determining the concentration of chlorite in environmental samples”
- 2 “Biological oxygen generation for space travel”
- 3 “Stimulation of organic contaminant degradation by the addition of chlorite”
- 4 “A novel treatment to prevent deep wound infection”
- 5 "A microassay for measurement of chlorite and perchlorate"
- 6 “Biological anaerobic treatment of BTEX contamination”

#### **National/international press:**

- 1 Perspectives Magazine (Oct. 1998)
- 2 Southern Illinoisan (Dec., 1998)
- 3 Daily Environment Report (Dec., 1998)
- 4 Chemical & Engineering News (Jan., 1999)
- 5 Industrial Bioprocessing Alert (Jan., 1999)
- 6 Technology catalysts (Apr., 1999)
- 7 Environmental Science & Technology News 4:515A (Dec. 1999)
- 8 US Congressional Press release (Aug. 2000)
- 9 University Press release (Sept. 2000)
- 10 Associated Press release (Sept. 2000)
- 11 Daily Egyptian (Oct 6. 2000)
- 12 Southern Illinoisan (Sept. 2000)
- 13 NPR-Chicago (Oct. 2000)
- 14 ABC News (Sept. 2000)
- 15 Voice of America Radio (Oct. 2000)
- 16 SIU Main web page (Oct. 2000)
- 17 American Society for Microbiology “Microbe World” web site (Nov. 2000)
- 18 Biocentuary Publications (July, 2001)
- 19 National Public Radio Boston “Living on Earth” (July, 2001)
- 20 Bloomberg News Wire Service (July, 2001)
- 21 Folha de Sao Paulo (Brazilian National Newspaper) (July, 2001)
- 22 The Globe and Mail (Canadian National Newspaper) (July, 2001)
- 23 Canadian Broadcasting Corp. “Information Morning” Radio Show (July, 2001)
- 24 CNN “Science and Technology Week” (June 2001)
- 25 WSIL Local TV News (July 2001)
- 26 The Guardian (British National Newspaper) (July, 2001)
- 27 Environmental Science and Technology News Update (July, 2001)
- 28 Popular Mechanics (Oct 2001, vol 178)
- 29 National Public Radio, Carbondale (Jan 2003)

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